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ETHICAL ANALYSIS OF NEW CHALLENGES AND INNOVATIVE APPROACHES IN WILDLIFE CONSERVATION

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ABSTRACT in English

Although inherently ethical, conservation enterprise requires conservationists to address challenging ethical issues. Addressing the problem of species extinction requires confronting the existence of multiple legitimate values of different stakeholders and sometimes also different perspectives on the conception of right and wrong actions. Conservationists must be able to highlight all critical issues arising from their projects and evaluate the various interests of different stakeholders so that the goals of their projects will be accepted and shared. Still, not all scientists in these disciplines are trained to identify and discuss the ethical issues arising from their work.

In this work are presented independent studies conducted applying tenets of conservation ethics both to develop and apply ethical tools to assess innovative approaches to conservation projects of critical endangered mammalian species and ethical issues arising from new challenges in wildlife conservation after the COVID-19 outbreak. Conservation ethics play a fundamental role in identifying and analyzing ethically relevant issues arising from conservation projects. Furthermore, it provides tools based on appropriate and articulated ethical principles to guide conservationists' decision-making.

Conservation projects need to make a thorough ethical evaluation from the conceptual stage of the proposal to its end, not only to respect the legal framework but also to assess the acceptability of the procedures and enhance the quality standards. To this aim, during this Ph.D. project, it was developed an ethical assessment tool (ETHAS) for assisted reproductive technologies (ARTs) and advanced assisted reproductive technologies (aARTs) applied in conservation projects. ETHAS was based on scientific literature, legislation, and ethical principles. The tool was then applied to assess the innovative approach that combines ARTs, which use natural gametes, with advanced ARTs, which use stem cell-associated techniques to produce in-vitro gametes, enacted by the scientists of the BioRescue project to save the northern white

rhinoceroses (*Ceratotherium simum cottoni*) from extinction. After its optimization performed in European zoos, ETHAS was applied to assess ART and aART performed by the scientist of the BioRescue: 1) at Ol Pejeta Conservancy, Kenya, for the ovum pick up (OPU) of the ova of the two living females of northern white rhinoceroses; 2) at Aventea, Cremona, Italy, for the IVF procedures; 3) at the laboratory of the Department of Stem Cell Biology at the Max Delbrück Center for Molecular Medicine, Berlin, Germany, for the procedure to generate iPSC from somatic tissue cells; 4) at the Department of Basic Medicine at Kyushu University, for procedures of in-vitro gametogenesis. ETHAS proved to be helpful in highlighting critical issues in the procedures and facilitating communication and discussion among the project partners about them. ETHAS outcome assessed the ART ovum pick-up and laboratory procedures as "totally acceptable" in the way they applied to rescue northern white rhinoceroses and these procedures have so far produced 26 viable embryos of northern white rhinoceroses. Whereas ETHAS assessed the aART procedures used to produce in-vitro gametes, starting from somatic cells as "Acceptable with mitigation", as they still require optimizations.

ART and aART represent powerful tools in the conservationists' toolbox for saving species from the verge of extinction, such as the northern white rhinoceroses, enhancing both in -situ and ex-situ conservation projects. Even if the final goal of conservation projects is to restore wild populations currently to be saved, threatened species still require ex-situ management in zoological facilities. Nowadays, zoos, aquaria, and other zoological facilities have taken prominent and active positions in endangered species conservation and educating visitors about the value of biodiversity. However, to be effective and trusted in their mission, these institutions must act ethically and have a good reputation among the general public. Drivers influencing a firm's reputation and the public's perception of how ethically correct it is acting are widely studied. Still, there are few studies focused on assessing the ethical reputation of zoological institutions.

During my Ph.D., I worked on the development of a tool for Assessing the Reputation of Zoos: The Zoo Ethical Reputation Survey (ZERS). ZERS is a survey designed with ad hoc items to analyze public opinion on features that can influence the reputation of a zoo, focusing on ethical aspects. After its development, ZERS was administrated to the visitors of two zoos in Italy and in Germany. It proved to be a tool able to provide information on the visitors' opinions about several drivers that, according to the literature, influence corporate reputation. It allowed the highlighting of some relations among the drivers; for example, it showed that visitors' opinions on zoos acting with ethical responsibility are correlated with the emotional appeal and familiarity they have with these institutions. Using ZERS can help zoos identify weaknesses in their reputation and develop new strategies to improve people's attitudes towards them, bringing many benefits to the individual zoo and zoological institutions in general.

The COVID-19 outbreak, among other things, has represented a big threat both to zoo's reputation since they had to face new, unexpected challenges and to the conservation of wildlife species. COVID-19 outbreak was immediately linked to a vast Chinese wet market selling live animals in Wuhan, China, and bats were soon suspected to be the reservoir of this new virus, with pangolins or civets as a potential intermediate reservoir. The way the media were framing news could substantially shape public risk perception, promoting or discouraging public tolerance towards these animals and wildlife in general. Together with an international team of scientists, during my Ph.D., I analyzed global media reports on bats before and during the pandemic across 26 countries and in 7 languages. Specifically, I worked on the dataset of the news collected in online newspapers in Portugal and Brazil. The gathered data showed that the overabundance of poorly contextualized reports on bat-associated diseases likely increased the persecution towards bats immediately after the COVID-19 outbreak. Our research provided ethical considerations for effective and correct conservation messaging regarding animals, such as bats, accused of being reservoirs of viruses but that also play a key ecological role for the

ecosystems and are at risk of extinction. Effective communication plays an essential role in adequately informing citizens on the ecological role of species such as bats and reassuring them about risks related to zoonoses. Still, giving correct scientific information might not be enough; the way information is shaped is substantial. Our research showed how relevant this was for bats. Soon after the first period following the COVID-19 outbreak, the intervention of various conservation communication initiatives allowed pro-conservation messages to resonate throughout the global media, stemming the increase in bat persecution.

While assessing the impact of the COVID-19 pandemic on bats, for which there are many in-situ conservation projects worldwide, it was also necessary to evaluate the effect of the pandemic on zoological institutions that play a relevant role in ex-situ conservation. After the COVID-19 outbreak, abruptly, zoos and aquariums had to deal with prolonged closure periods due to lockdown and other restrictive measures adopted to reduce the spread of SARS-CoV-2, the etiologic agent of the disease. The workers of these institutions faced new challenges to maintaining the high standards of their work and animals' welfare at the same level as pre-pandemic. Conservation programs, research, integrated conservation projects, and educational programs were affected, too. The sudden absence of visitors directly impacted the revenues, and zoological institutions had to implement new strategies to engage the public in their activities. To assess the public's awareness of the impact of the pandemic on zoological institutions, during my Ph.D., I worked with researchers of the Ethics Laboratory for Veterinary Medicine, Conservation and Animal Welfare of Padua University and the Unione Italiana dei Giardini Zoologici ed Acquari, to develop a survey based on a questionnaire for the public. The questionnaire was uploaded on LimeSurvey platform, and the link to the survey was disseminated via social media. As people are more likely to answer questionnaires if directly involved, the public's questionnaire was also administered directly by researchers to visitors of several Italian zoological institutions. Results of the survey highlighted that the public is aware

of the negative economic impact on zoological institutions of lockdown and other periods of restriction of movement. According to the respondents, the pandemic severely affected the promotion of scientific knowledge and environmental education activities, and there was a lack of public support for zoos and aquariums in those difficult times.

In all these research works, during my Ph.D., I worked on several different ethical aspects arising in different conservation contexts by applying conservation ethics to highlight the specific ethical issues that were continuously arising and developing and implementing ethical tools that can help in the ethical review processes of conservation practices.

ABSTRACT in italiano

Sebbene l'impegno per la conservazione sia etico di per sé, è necessario che i conservazionisti siano pronti ad affrontare anche le impegnative questioni etiche che ne derivano. Affrontare il problema dell'estinzione delle specie richiede un confronto con l'esistenza di molteplici valori legittimi di diverse parti interessate e, talvolta, anche con prospettive diverse sulla concezione di quali siano azioni le giuste e quelle sbagliate. I conservazionisti devono saper evidenziare tutte le criticità derivanti dai loro progetti e saper valutare i vari interessi delle diverse parti interessate, affinché gli obiettivi dei loro progetti siano accettati e condivisi. Tuttavia, ancora, non tutti gli scienziati di queste discipline sono formati per identificare e discutere le questioni etiche derivanti dal loro lavoro.

Durante questo periodo di dottorato, ho effettuato diversi lavori indipendenti, che hanno sfruttato i principi dell'etica della conservazione al fine di sviluppare e applicare strumenti etici per la valutazione di approcci innovativi alla salvaguardia di specie di mammiferi a rischio di estinzione che sfruttano le biotecnologie e per analizzare le questioni etiche derivanti dalle nuove sfide che si sono presentate nella conservazione della fauna selvatica, dopo l'insorgere della pandemia di COVID-19. L'etica della conservazione svolge un ruolo fondamentale nell'identificazione e nell'analisi delle questioni eticamente rilevanti derivanti dai progetti di conservazione. Inoltre, fornisce strumenti basati su principi etici appropriati e articolati per guidare le decisioni dei conservazionisti.

È necessario che i progetti di conservazione siano sottoposti a una valutazione etica approfondita fin dalla prima fase concettuale della proposta e in tutte le altre fasi fino alla sua conclusione, non solo per rispettare il quadro giuridico, ma anche per valutare l'accettabilità delle procedure proposte e migliorare gli standard di qualità in genere. A questo scopo, durante il presente progetto di dottorato, è stato sviluppato uno strumento di valutazione etica (ETHAS) per le tecnologie di riproduzione assistita (ARTs) e le tecnologie avanzate di riproduzione assistita

(aARTs) applicate nei progetti di conservazione di specie di mammiferi a rischio di estinzione. ETHAS è stato sviluppato basandosi sulla letteratura scientifica, sulla legislazione e sui principi etici. Lo strumento è stato poi applicato per valutare l'approccio innovativo proposto dagli scienziati del progetto BioRescue per salvare i rinoceronti bianchi del Nord (*Ceratotherium simum cottoni*) dall'estinzione. Questo innovativo approccio combina le ART, che utilizzano gameti naturali, con le ART avanzate, che utilizzano tecniche associate alle cellule staminali per produrre gameti in vitro. Dopo la sua ottimizzazione effettuata in zoo eupopei, lo strumento è stato applicato per valutare le procedure di ART e aART eseguite dagli scienziati del BioRescue presso la Ol Pejeta Conservancy, in Kenya, dove avviene il prelievo di ovociti (OPU) dalle ultime due femmine viventi di rinoceronte bianco del Nord; presso Aventea, a Cremona, in Italia, dove vengono messe in atto le procedure di fecondazione assistita in vitro con gameti naturali; presso il laboratorio del Dipartimento di Biologia delle Cellule Staminali del Max Delbrück Center for Molecular Medicine, a Berlino, in Germania, dove vengono effettuate le procedure per produrre iPSC da cellule tissutali; presso il Dipartimento di Medicina di Base dell'Università di Kyushu, dove vengono effettuate le procedure per gametogenesi in vitro. ETHAS si è rivelato utile per evidenziare le criticità delle procedure e facilitare la comunicazione e la discussione tra i partner del progetto. L'esito di ETHAS ha valutato le procedure di prelievo degli ovociti e le procedure di laboratorio per la fecondazione in vitro, come procedure "totalmente accettabili" nel modo in cui sono state applicate per il salvataggio dei rinoceronti bianchi del Nord e, ad oggi, hanno prodotto 26 embrioni vitali del rinoceronte bianco del Nord. ETHAS ha, invece, valutato le procedure aART utilizzate per produrre gameti in vitro, partendo da cellule somatiche, come "Accettabili dopo aver messo in atto azioni di mitigazione dei potenziali rischi", in quanto richiedono ancora ottimizzazioni.

ART e aART rappresentano strumenti con enormi potenzialità nella “cassetta degli attrezzi” dei conservazionisti per salvare specie sull'orlo dell'estinzione, come i rinoceronti bianchi Nord, e

sono in grado di rendere i progetti di conservazione sia in in-situ che ex-situ molto più efficaci. Anche se l'obiettivo finale dei progetti di conservazione è quello di ripristinare le popolazioni selvatiche, attualmente le specie minacciate, per poter essere salvate, richiedono molto spesso una fase di gestione ex-situ nelle strutture zoologiche. Oggi gli zoo, gli acquari e le altre strutture zoologiche hanno assunto posizioni di rilievo e attive nella conservazione delle specie minacciate e nell'educazione dei visitatori sul valore della biodiversità. Tuttavia, affinché queste istituzioni siano efficaci e affidabili nella loro missione devono dimostrare che stanno agendo in modo etico e devono godere di una buona reputazione dell'opinione pubblica. I fattori che influenzano la reputazione di un'azienda e la percezione che il pubblico ha su quanto eticamente essa si stia comportando sono ampiamente studiati in letteratura scientifica. Tuttavia, sono pochi gli studi incentrati sulla valutazione della reputazione etica delle istituzioni zoologiche.

Durante il mio dottorato, ho lavorato allo sviluppo di uno strumento per valutare la reputazione degli zoo: Lo Zoo Ethical Reputation Survey (ZERS). Lo ZERS è uno strumento che, attraverso un sondaggio progettato con item ad hoc, analizza l'opinione pubblica sulle caratteristiche che possono influenzare la reputazione di uno zoo, concentrandosi in particolare sugli aspetti etici. Dopo lo sviluppo dello strumento, ZERS è stato somministrato ai visitatori di due zoo, uno in Italia e uno in Germania. Esso si è rivelato uno strumento in grado di fornire informazioni sull'opinione dei visitatori in merito a diversi fattori che, secondo la letteratura, influenzano la reputazione aziendale. Ha permesso di evidenziare alcune interessanti relazioni tra i driver; ad esempio, ha mostrato che le opinioni dei visitatori su quanto gli zoo agiscono eticamente sono correlate al richiamo emotivo che queste istituzioni sanno suscitare in loro e al livello di familiarità che hanno con queste istituzioni. L'utilizzo di ZERS può aiutare i giardini zoologici a identificare i punti di debolezza che possono influenzare la loro reputazione e a sviluppare nuove strategie per migliorare l'atteggiamento delle persone nei loro confronti, portando molti benefici non solo al singolo giardino zoologico, ma alle istituzioni zoologiche in genere.

L'epidemia di COVID-19 ha causato, tra le altre cose, una situazione di rischio per la reputazione delle istituzioni zoologiche, che si sono travati ad affrontare nuove e inaspettate sfide, e per la conservazione della fauna selvatica. L'epidemia di COVID-19 è stata immediatamente collegata a un vasto mercato cinese di animali vivi a Wuhan, in Cina, e i pipistrelli sono stati presto sospettati di essere il serbatoio di questo nuovo virus, con pangolini o zibetti come potenziali serbatoi intermedi. In questa particolare situazione, il modo in cui venivano pubblicate le notizie dai media, avrebbe potuto influenzare in modo sostanziale la percezione del rischio nelle persone, promuovendo o scoraggiando la tolleranza verso questi animali e la fauna selvatica in generale. Insieme a un team internazionale di scienziati, durante il mio dottorato ho analizzato gli articoli pubblicati dai media on line sui pipistrelli prima e durante la pandemia in 26 paesi e in 7 lingue. In particolare, ho lavorato sul dataset delle notizie raccolte dai giornali online in Portogallo e Brasile. I dati raccolti hanno dimostrato che la sovrabbondanza di notizie non ben contestualizzate sulle malattie associate ai pipistrelli ha molto probabilmente aumentato la persecuzione nei confronti di questi animali subito dopo l'epidemia COVID-19. La nostra ricerca ha fornito considerazioni etiche per un'efficace e corretta comunicazione sulla conservazione di animali, come i pipistrelli, accusati di essere serbatoi di virus, ma che svolgono anche un ruolo ecologico fondamentale per gli ecosistemi e sono già a rischio di estinzione. Una comunicazione efficace svolge un ruolo essenziale nell'informare adeguatamente i cittadini sul ruolo ecologico di specie come i pipistrelli e nel rassicurarli sui rischi legati alle zoonosi. Tuttavia, fornire notizie scientificamente corrette potrebbe non essere sufficiente. Il modo in cui le informazioni vengono formulate è fondamentale e la nostra ricerca ha dimostrato quanto questo sia stato importante per i pipistrelli. Nel primo periodo immediatamente successivo all'epidemia di COVID-19, le varie iniziative di comunicazione messe in atto da parte di scienziati e conservazionisti hanno permesso di divulgare, nei media di tutto il mondo, messaggi a favore della conservazione dei pipistrelli ed è stato evitato il possibile aumento di azioni contro questi animali.

Così si è ritenuto importante valutare l'impatto della pandemia COVID-19 sui pipistrelli, che sono oggetto di numerosi progetti di conservazione in-situ in tutto il mondo, allo stesso modo si è ritenuto importante valutare anche l'effetto della pandemia sulle istituzioni zoologiche che svolgono un ruolo rilevante nella conservazione ex-situ. Dopo l'esplosione della COVID-19, improvvisamente, zoo e acquari hanno dovuto affrontare prolungati periodi di chiusura a causa delle misure di blocco e di restrizione adottate per ridurre la diffusione dell'agente eziologico della malattia, il SARS-CoV-2. I lavoratori delle istituzioni zoologiche hanno dovuto affrontare impreviste nuove sfide per poter mantenere gli elevati standard del loro lavoro e il benessere degli animali allo stesso livello di prima della pandemia. Anche i programmi di conservazione, la ricerca, i progetti di conservazione integrata e i programmi educativi ne hanno risentito. L'improvvisa assenza di visitatori ha avuto un impatto diretto sulle entrate e le istituzioni zoologiche hanno dovuto implementare nuove strategie per coinvolgere il pubblico nelle loro attività. Per valutare la percezione degli operatori dell'impatto della pandemia sul loro lavoro e la consapevolezza del pubblico su questi argomenti, durante il mio dottorato ho collaborato con i ricercatori del Laboratorio di Etica di Medicina Veterinaria, Conservazione e Benessere Animale dell'Università di Padova e dell'Unione Italiana dei Giardini Zoologici ed Acquari, per sviluppare un'indagine basate su un questionario da somministrare al pubblico. Il questionario è stato, poi, caricato sulla piattaforma LimeSurvey e il link è stato diffuso tramite i social media. Poiché le persone sono più propense a rispondere ai questionari se coinvolte direttamente, il questionario per il pubblico è stato somministrato anche direttamente dai ricercatori ai visitatori di diverse istituzioni zoologiche italiane. I risultati dell'indagine hanno mostrato che le persone intervistate erano consapevoli che il lockdown e gli altri periodi di restrizioni di movimento della popolazione hanno avuto un impatto negativo sulle istituzioni zoologiche. Secondo gli intervistati, la pandemia ha colpito gravemente anche la promozione della conoscenza scientifica

e le attività di educazione ambientale e che c'è stata una mancanza di sostegno pubblico per gli zoo e gli acquari in quel difficile periodo.

Nel corso di tutti questi lavori di ricerca, durante il mio dottorato, ho lavorato su diversi aspetti etici che emergono in diversi contesti di conservazione della fauna selvatica, applicando l'etica della conservazione per evidenziare le questioni specifiche che si presentavano continuamente e sviluppando e implementando strumenti etici che possono aiutare nei processi di revisione etica delle pratiche di conservazione.

GENERAL INTRODUCTION

Earth is facing the sixth mass extinction (Barnosky et al., 2011). The accelerated human-induced loss of biodiversity not only threatens valuable ecosystem services but can also negatively affect human well-being (Ceballos et al., 2015). Twenty-seven percent of wild mammal species are threatened with extinction (IUCN). At the same time, humans and their domesticated animals account for about 30 times the living mass of all wild mammals and compete with wildlife for space and resources (Bar-On et al., 2018). Many scientists advocate that it is a scientific and moral imperative to take rapid action to stop this extinction because the magnitude and possible impacts on humans are only partially predictable (Bradshaw et al., 2021; Ceballos et al., 2020). For instance, according to the World Health Organization, biodiversity loss is a global threat to human existence, affecting economies, societal equality, way of life, and health (Lawler et al., 2021; WHO, 2015).

The development and implementation of habitat protection policies and habitat restoration projects are the first and most significant steps in conserving biodiversity for future generations (Tilman et al., 2017). However, this might no longer be sufficient to save biodiversity, as many species are now fragmented, resulting in non-viable populations with low genetic diversity (Comizzoli, 2015; Hoban et al., 2020). In these cases, conservation biology, which takes advantage of advanced laboratory biotechnologies to save endangered species, can be the last chance. Assisted reproduction techniques (ART) — such as assisted insemination (AI) or in-vitro fertilization (IVF) — are becoming essential in many conservation projects representing an efficient tool in the conservationist's toolbox. Still, for some species with already too few individuals alive, ART can no longer be sufficient to maintain genetic diversity for the population's long-term sustainability. For this species, advanced assisted reproduction technologies (aART), which use somatic cells to generate in-vitro gametes, may offer a glimmer of hope for being saved. ART and aART increase the chances of successful conservative breeding programs by overcoming infertility and optimizing genetic management, avoiding inbreeding (or outbreeding) depression, and risks of inherited disease transmission (Comizzoli

& Holt, 2019; Herrick, 2019). Though, to be successful, they require knowledge of the reproductive biology of each species to which they are to be applied, which is often not yet available (Herrick, 2019). Besides, such knowledge may be difficult to achieve in endangered species because of the limited number of individuals available for study and potential access difficulties (Comizzoli, 2015). In attempting to obtain such knowledge, conservationists face the dilemma of investing in money, time, and personnel and using the last precious individuals to save the species or just protecting the last remaining individuals waiting for the inevitable (Monfort, 2014). This was the fate of George (*Achatinella apexfulva*), who died in 2019, Benjamin (*Thylacinus cynocephalus*), who died in 1936, and Martha (*Ectopistes migratorius*), who died in 1914, to name but a few cases of last individuals of species recently extinct. For many species or subspecies on the brink of extinction or even functionally extinct — such as northern white rhinoceroses (*Ceratotherium simum cottoni*), which has only two extant individuals, both females — aARTs remain the last hope for increasing genetic variability for future generations (Ryder et al., 2020). At the same time, for all the species for which scientists are currently unable to develop successful breeding projects or species with a declining number of individuals, it is essential to collect as many samples and gametes as possible to be safeguarded in biobanks to preserve biodiversity for future generations (Byers et al., 2013; Comizzoli, 2017; Holt & Comizzoli, 2021).

Earth has entered an era of rapid biodiversity decline. Many species have very limited, fragmented populations in the wild. Isolated populations have little or no genetic exchange, and mating of closely related animals increases homozygosity and inbreeding depression, which is the cause of transmission of hereditary diseases and fertility problems and higher disease susceptibility that increase extinction risk (Acevedo-Whitehouse et al., 2003; Comizzoli, 2016; Roldan et al., 2006). In such cases, in situ conservation alone will no longer be effective, and ex-situ conservation can play a crucial role. The conservation value and potential of ex-situ management have long been known and described as ‘Ark Concept’ (Bowkett, 2009). In ex-situ conservation, the threatened species are kept in captivity until they can be safely reintroduced,

and this approach has successfully restored the population of several species into their native habitats, such as California condor (*Gymnogyps californianus)* and Arabian oryx (*Oryx leucoryx*), to name a few (Bowkett, 2009). However, also in ex-situ conservation projects, it is often necessary to incorporate assisted reproduction technologies (ART) into classical zoo breeding programs. Assisted reproductive technologies that use natural gametes are well-established in farm animals, and these protocols can be used after adaptation for wild close-related species. For example, the rhinoceros and horse share a common ancestor; therefore, assisted reproduction techniques (ART) developed in equines can potentially be translated to rhinoceros species (Hildebrandt et al., 2018). In addition to classical ART, recent advancements in scientific knowledge have led to advanced ART (aART) that will help breeding projects be ever more efficient. The term advanced assisted reproduction technologies (aART) was used for the first time in 2004 (Baldassarre & Karatzas, 2004). The term aART refers to more futuristic approaches that use recent advances in biotechnology and stem-cell-related approaches such as cloning, inner cell mass transfer (ICM), and stem-cell-associated techniques (SCAT) for in-vitro generation of gametes and embryos.

In figure n.1 are represented several different strategies that can be potentially applied to save critically endangered species, such as the northern white rhinoceroses.

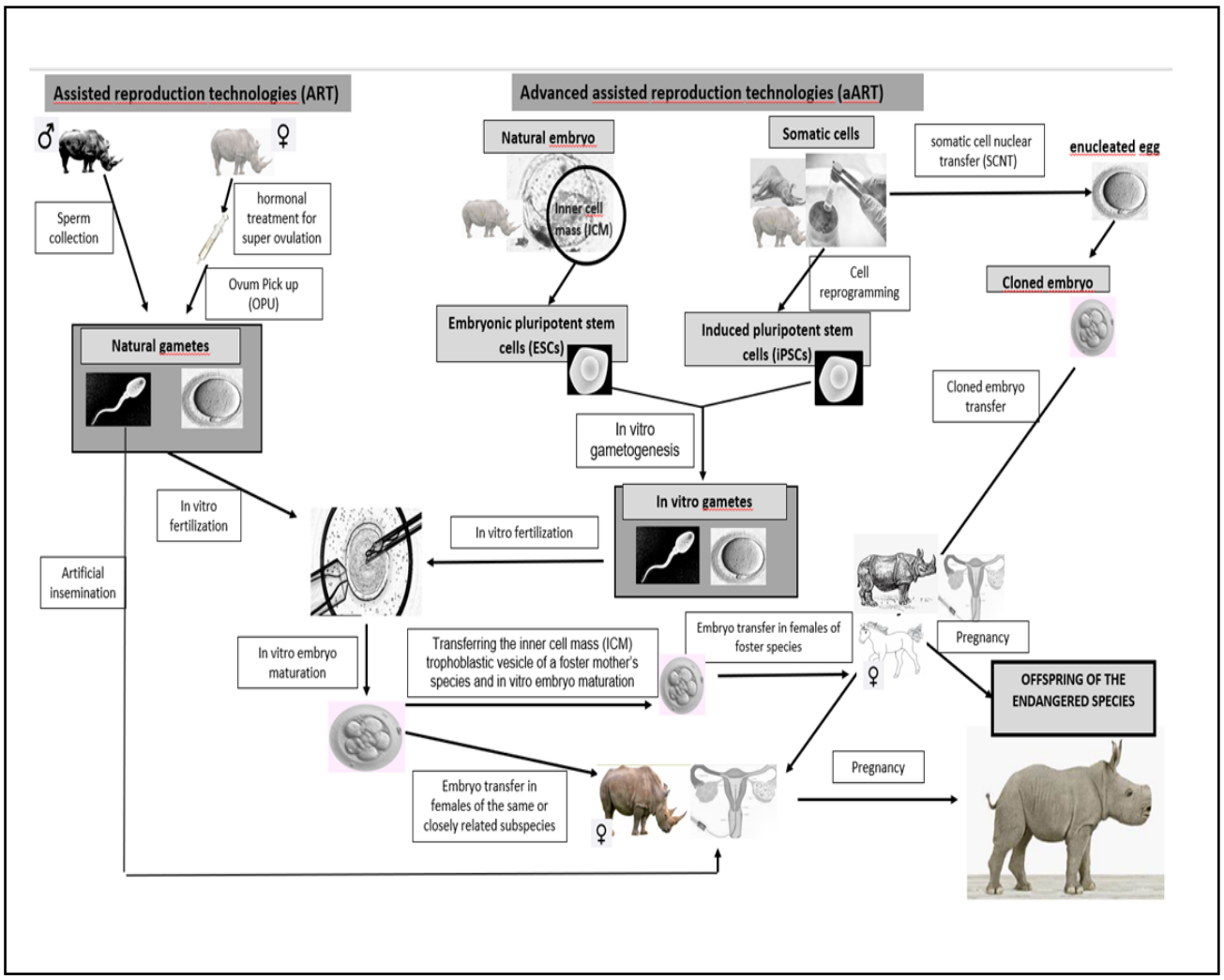

Fig. 1 Assisted reproduction technologies (ART) and advanced assisted reproduction technologies (aART) that can be applied to save critically endangered species.

Assisted reproductive technologies that use natural gametes, such as artificial insemination (AI) and in vitro fertilization, are, until now, the only reproductive technologies to be successfully applied to wildlife conservation. Artificial insemination (AI) has helped save species such as the giant panda (*Ailuropoda melanoleuca*) and the black-footed ferret (*Mustela nigripes*) from extinction. It has also been successfully applied to southern white rhinoceroses (*Ceratotherium simum simum*) and has already produced viable offspring in more than 50 wildlife species

(Hermes et al., 2009; Hildebrandt et al., 2007; Mastromonaco & Songsasen, 2020). Though, artificial insemination cannot be used for many species, with few remaining individuals that cannot have viable pregnancies due to health and age problems, as in the case of the northern white rhinoceroses (De Mori et al., 2021; Saragusty et al., 2016). In cases like this, gametes collection, in-vitro fertilization, and embryos transfer into the uterus of a recipient mother of a closely related taxon can over the problem (Saragusty et al., 2016). The first important step is gametes collection, which should be done whenever possible (Comizzoli et al., 2022). Several methods have been developed for semen collection from wild animals, but one of the most used is electroejaculation and urethral catheterization (Hildebrandt et al., 2021). The oocyte pick-up (OPU) is more invasive and complex. It requires ultrasound-guided probes that need to be optimized for the specific anatomy of the species to be used transvaginally or transrectally. Oocyte collection from alive-wild animals has rarely been applied in conservation projects (Mastromonaco & Songsasen, 2020). However, recently, it has been successfully repeatedly performed on the two surviving females of northern white rhinoceroses, which showed to recover quickly from the procedure, and 119 oocytes were retrieved in eight procedures between 2019 and 2022 (Biasetti et al., 2022; BioRescue, 2023.; de Mori et al., 2021; Hildebrandt et al., 2018). Additionally, gametes and gonadal tissues can be collected from deceased individuals (Hildebrandt et al., 2021).

Natural gametes can be used for in vitro fertilization (IVF). Embryos can be produced in vitro by introducing sperm close to the oocyte and allowing natural fertilization. Alternatively, to promote fertilization, intracytoplasmatic sperm injection (ICSI) can be used, and sperm may be injected directly into the eggs. ICSI is successfully applied to domestic and farm animals but rarely to wild animals and requires expensive equipment and expertise; therefore, it can be considered an aART in wildlife reproduction technologies (Galli et al., 2014; Hermes et al., 2009; Hildebrandt et al., 2021). In-vitro fertilization offers significant advantages: it can allow the reproduction of individuals that do not mate naturally and the sorting of the sperm used to decide the sex of the newborns (Hildebrandt et al., 2021).

The embryos obtained by natural gametes can be cryopreserved in biobanks or transferred to the uterus of a recipient mother. The recipient mother can be of the same species or a close-related, not-endangered subspecies (Saragusty et al., 2016). The last is the case with northern white rhinoceroses. The two remaining females, Najin and Fatu, cannot have viable pregnancies due to health and age problems. However, it is still possible to pick up their eggs and fertilize them in vitro with the sperm of five now-dead males whose gametes scientists have collected and cryopreserved in different biobanks in the past decades. The embryos can be transferred to recipient mothers of the close-related subspecies of southern white rhinoceroses to achieve a new generation of northern white rhinoceroses.

To save critically endangered species, ART that uses only natural gametes might not be sufficient due to the fact that the remaining individuals are often closely related. To increase genetic variability, scientists should take advantage of each technique available in assisted reproductive technologies that could help in the genetic rescue of the species (Saragusty, Ajmone-Marsan, et al., 2020). Genetical rescue is defined as the immigration of unique genomes into a population to restore genetic diversity, increasing its absolute fitness, measured by an increase in population size or growth (Sandler et al., 2021; Whiteley et al., 2015)

Somatic cell nuclei of cells of endangered species are introduced into the enucleated oocytes. This was the first advanced assisted reproduction based on biotechnologies to be applied in animal conservation. Then, the reconstructed SCNT oocytes are artificially activated to initiate a developmental program to form blastocysts that can be transferred to the uterus or recipient mothers. In interspecific somatic cell nuclear transfer (iSCNT), the nuclei of endangered species cells are transplanted into an enucleated egg of a not endangered species (Lanza et al., 2000). The first pioneering studies of SCNT were conducted in 1958 by Gurdon on amphibians (Gurdon et al., 1958). In 1996 it was successfully used on mammals to obtain a cloned sheep, Dolly (Campbell et al., 1996). Since then, successes in cloning at least 20 mammalian species have been reported (Matoba & Zhang, 2018). In 2000, it was obtained the first cloned wild animal:

Noah, a gaur (*Bos gaurus*), a wild Bovidae listed as Vulnerable on the IUCN Red List since 1986 (Lanza et al., 2000).

However, despite some successes, several technical hurdles have limited the practical use of SCNT technology. First, the cloning efficiency is extremely low in essentially all species. Second, abnormalities are frequently observed in the extraembryonic tissues, such as the placenta, of the cloned embryos, and a high fetal death rate (Folch et al., 2009; Hildebrandt et al., 2021; Matoba & Zhang, 2018). Moreover, generally, this technology doesn't increase the genetic biodiversity of the species, as it generates animals that are genetically identical to another individual. However, SCNT has been successfully applied to restore black-footed ferret species (*Mustela nigripes*). The black-footed ferret is an endangered species saved from extinction by an ex-situ breeding program of the last 18 animals found alive in the wild, of which only seven produced offspring. Because of the small founding population size, the species exhibit signs of inbreeding depression (Wisely et al., 2015). Nevertheless, in 2020, a healthy black-footed ferret cloned from 30-year-old fibroblasts of a never reproduced animal was born, bringing back this unique genome into the gene pool of the species (Sandler et al., 2021). Using SCNT in this way has helped enrich the species with an unrepresented genotype. SCNTs can also be used to generate embryos of a target species from which to collect inner cell mass (IMC) (Saragusty, Ajmone-Marsan, et al., 2020). ICMs can then be used for in-vitro gametogenesis to create more genetic variability from only one genotype.

In-vitro gametogenesis associated with stem technologies has opened a new promising path for the genetic restoration of endangered species. Thanks to meiosis, this approach can generate an enormous variety of new genotypes by reshuffling existing diversity through chromosome reassortment. By using in-vitro-generated gametes, the gene pool diversity of the species could be further enriched by crossbreeding in vitro not only alive individuals but also now-dead individuals. This can be obtained thanks to the in-vitro-generated gametes produced from iPSCs obtained from different types of tissues cryopreserved in biobanks. Furthermore, stem-associated technologies (SCATs) can help overcome many limitations, hitherto considered insurmountable,

to save a species, such as creating oocytes from iPSCs of male donors after silencing Y-chromosome-related genes (Hildebrandt et al., 2021; Ledford & Kozlov, 2023).

Stem-associated technologies (SCATs) can use iPSC that, similarly to embryonic stem cells (ESCs) can grow indefinitely while maintaining pluripotency and are capable of differentiating into all three germ layers (Takahashi & Yamanaka, 2006). However, unlike ESCs, iPSCs do not require embryonic tissues for harvesting but can be produced by reprogramming somatic cells. The iPSC has been successfully established for several domestic and laboratory species, but also for endangered species, such as the snow leopard, and critically endangered species, such as the northern white rhinoceros (Bank et al., 2021; Friedrich Ben-Nun et al., 2011; Korody et al., 2021; Verma et al., 2012; Zywitza et al., 2022).

The most widely applied approach to creating iPSC lines is to use Yamanaka reprogramming factors OCT4, SOX2, KLF4, and cMYC (Stanton et al., 2019; Takahashi & Yamanaka, 2006). Further research on species-specific reprogramming factors is needed to overcome the difficulties in producing stable transgene-free iPSCs (Stanton et al., 2019). However, recently, improved and reproducible methods have been applied to produce iPSCs from northern white rhinoceros cells that allow the production of transgene-free iPSCs that do not require a continued expression of exogenous reprogramming factors to maintain pluripotency and that, therefore, can be used for gametogenesis in vitro (Korody et al., 2021; Zywitza et al., 2022).

Given their pluripotency, induced pluripotent stem cells, embryonic stem cells (ESCs) from cloned embryos, and ESCs from in vitro fertilization (IVF) embryos, can differentiate into all different embryo tissues, including the germ cell lineage. Therefore, under appropriate conditions that can be recreated in vitro, the germ cell lineage can mature into gametes. This procedure is called in-vitro gametogenesis. All germ cell lineages originate from primordial germ cells (PGCs), which are segregated from the somatic cell lineage at an early developmental stage when a characteristic gene expression program appending genome-wide epigenetic change is observed in epiblast cells heading to PGCs (Hayashi & Saitou, 2014).

The research in murine models shows that the germ cell lineage is derived from the pluripotent cell population in response to extrinsic signals. Evidence from genetic studies has uncovered the extrinsic signals essential for PGC specification, and researchers think they could also be used for other mammals (Hayashi & Saitou, 2014). However, there are distinct types of pluripotent states with respect to the responsiveness to extrinsic signals. Studies in vivo have revealed that it is likely that ESCs acquire PGC-competence during conversion from the naïve to primed pluripotent state (Hayashi & Saitou, 2014). Therefore, the induced pluripotent stem cell to be used in in vitro gametogenesis must reach the naïve pluripotent state (Hayashi & Saitou, 2014; Zywitza et al., 2022). The following step is the reconstruction in vitro of the PGCs specification processes to convert the naïve state to an epiblast-like state with PGC-competence, the PGC-like cells (PGCLCs), under a defined set of conditions and extrinsic signals (Hayashi et al., 2011). The PGCLCs have a similar pattern of gene expression and genome-wide reorganization of epigenetic modification similar to that of PGCs in vivo (Hayashi & Saitou, 2014).

The final step is represented by the differentiation of PGCLCs into spermatozoa and oocytes. The process in vivo depends on the environment of the gonads, testis, and ovary, respectively (Hayashi & Saitou, 2014).

In vitro, the environment required for the sexual differentiation of PGCLCs in germ cells, is achieved by co-culture of PGCLCs with embryonic gonadal somatic cells (Hikabe et al., 2016; Ishikura et al., 2016). However, for in-vitro gametogenesis in the context of endangered species, the fetal gonadal somatic must be very difficult to obtain. In these cases, xeno-reconstituted ovaries with mouse fetal gonadal somatic cells would be one option to bypass this obstacle (Hayashi et al., 2021). Although partially positive results have been reported for the in vitro generation of germ cells in a number of mammalian livestock species, currently, mice remain the only species for which germ cell development has been fully reconstituted in vitro (Hayashi et al., 2017; Hikabe et al., 2016; Ishikura et al., 2016).

Finally, even if robust methodologies for generating gametes in vitro from endangered species are achieved in the medium term, the next step to overcome will be to have sufficient numbers

of potential recipient females to carry the created embryos to term (Saragusty, Ajmone-Marsan, et al., 2020). An alternative solution could be recruiting females from non-endangered species (Saragusty et al., 2016; Saragusty et al., 2020). To avoid abortion, it is necessary to trick the foster mother's immune system into believing it carries an embryo of its kind (Saragusty et al., 2020). This can be achieved by transplanting the ICM of the endangered species, which are the cells that will form the fetus, into the trophoblastic vesicles that will give the placenta, obtained by removing the ICM from an embryo of the foster mother species (Saragusty et al., 2016; Saragusty et al., 2020). However, this is not the case for endangered species such as the northern white rhinoceroses, for which the close subspecies of southern white rhinoceroses could be used to obtain gonadal for in vitro gametogenesis and recipient mothers (Saragusty et al., 2016).

Additionally, biobanks play a key role in ART and aART. The Frozen Zoo®, for example, is the largest and most diverse collection of living cell cultures, oocytes, sperm, and embryos representing nearly 1,000 different taxa (*Frozen Zoo*®). More institutions like this one should be established to store samples of particularly at-risk taxa in several different biobanks for safety reasons.

Long-term preservation of gametes, embryos, and tissues allows conservationists to overcome problems related to space and time. Preserved specimens can be easily transferred from a facility or the wild to the laboratories or other facilities, and vice versa, without translocating the animals. In addition, preserved specimens can be used in the future, eliminating the time boundary, and the genome of long-deceased individuals can be reintroduced into the species' gene pool whenever necessary (Saragusty et al., 2016). Various biomaterials can be preserved using cryopreservation in liquid nitrogen at −196 °C for extended periods of time after being added with cryoprotectants. However, in many cases, cryopreservation of gametes (especially oocytes, which often have low permeability to cryoprotectants), embryos, or tissues could be challenging (Woods et al., 2004). Additionally, long-term liquid nitrogen storage is costly, and sample translocation requires special care. To overcome these problems, researchers attempted a different strategy applying desiccation to long-term preserving sensitive mammalian cells in the

dry form. Biobanking and maintaining the desiccated samples at ambient temperatures would reduce costs and make sample transportation simple and cheap. Furthermore, this technology could make biobanking accessible to developing countries with unreliable liquid nitrogen and/or power supply. Still, because gametes, cells, and tissues of higher organisms can die when drying exceeds a certain threshold, further studies will have to be conducted to protect them during the drying and rehydration processes before applying this promising technology to biobanking (Saragusty et al., 2016).

All these biotechnologies enhance the possibility of increasing the number of individuals, also allowing a genetic rescue of species on the verge of extinction. Some of them have already been successfully applied, and others are under optimization for wild animals, but their application to projects such as the one proposed by BioRescue is very promising.

In April 2022, the scientist of BioRescue announced that they succeeded in producing iPS cells from the cryopreserved tissue of Nabire – a now death northern white rhinoceros –using the method of episomal reprogramming (BioRescue). They introduced genes that reprogram the skin cells into iPS cells into the genome of the skin cells using plasmids (Zywitza, V. et al., 2022). They obtained cells in naïve state – the "original state" of pluripotency that can be used to produce germline cells. The transcriptome of the cells has been characterized. These first iPS cells of northern white rhinoceroses cannot be used for in-vitro gametes production because the karyogram revealed that the cells had $2n = 81$ chromosomes due to aneuploidy of fibroblasts of Nabire (Houck M.L. et al., 1994; Tunstall, T. et al., 2018; Zywitza, V. et al., 2022). Nevertheless, it represents a significant advancement in the scientific knowledge toward the possibility of generating germline cells of rhinoceroses. It will help all the Rhinocerotidae family, as all five extant species that currently are at risk of extinction**.**

However, all these procedures are time and money-consuming, and opinions differ on whether human involvement in the conservation of species on the verge of extinction is desirable and what constitutes "good" and "bad" human interventions concerning wildlife. The use of these biotechnologies applied to conservation can be perceived as unnatural. Furthermore, they might

see the can be seen as a case of "technofix", the use of biotechnology to reverse the outcomes of morally problematic activities (e.g., habitat loss, etc.), leaving intact the causes (Saragusty J., 2012).

Wildlife protection generates ethical disagreements and dilemmas in which human needs, preferences, and interests, concern for individual animal welfare, and the value of biodiversity, ecosystems, and wild nature are part of the discussion (Paquet et al., 2010). There is no specific reason to justify the efforts and prioritize the value of ethical conflicts arising from conservation projects. According to Biasetti et al. (2016), it is necessary to adopt a complex framework of values: the "economic value of nature" that justify the conservation of nature for economic reasons; the "ecological value of nature" that justifies the conservation as essential services for the survival of life on our planet provided by ecosystems; the "flourishing value of nature" that justifies conservation because of nature provides important "intangible" necessities – wellbeing, beauty, knowledge, and autonomy– relevant for human thriving.

In the first part of section 1 of this Ph.D. dissertation, I present the development of a frame for the ethical analysis of the application of assisted reproduction technologies in biodiversity conservation projects. The decision-making process on using ART and aART in conservation projects can be very complex, dealing with various values and potential ethical issues. It is essential to assess goals, the probability of achieving them, and the values they convey. The procedure must be conducted within a recognized legal framework and to the best standards in order to protect animals, ecosystems, and scientists and to maintain public confidence.

Additionally, an analysis of the potential benefits, risks, and costs associated with the procedure itself is essential. Potential benefits, risks, and costs should be evaluated for all the stakeholders involved in the procedures for an overall evaluation of the procedure.

To build a frame for the analysis of the use of ART and aARTs in conservation projects, in the present work, it was used an ethical matrix applied to procedures proposed by the BioRescue to save the northern white rhinoceroses from extinction. This ethical tool was developed by Mepham (1996), and has already been applied to many fields, including veterinary medicine

738 (England and Millar, 2008) and the assessment of human-animal interactions (de Mori et al.,
739 2019; Biasetti et al., 2020). In previous work, the ethical matrix had been adapted to the ethical
740 analysis in conservation (Biasetti and de Mori, 2016).
741 The ethical matrix applied to the decision-making process in conservation projects allows us to
742 unpack and analyze the ethically relevant aspects necessary for the decision-making. It helps
743 organize all the relevant aspects of the three categories of potential stakeholders: ecological
744 entities, individual animals, and people, and highlights the ethical tenets involved, such as
745 wellbeing, autonomy, and fairness for each stakeholder.
746 The proposed frame was then applied to develop an ethical tool for assessing specific procedures
747 of assisted reproduction technologies in wildlife breeding programs: the ethical assessment tool
748 (ETHAS). In section 1.2, it is presented the development and application of ETHAS to the ovum
749 pick-up procedures that have been applied to Najin and Fatu.
750 The development ETHAS was based on the scientific literature, on a review of legislation and
751 international treaties, and integrated with the ethical principles and values highlighted in the
752 previous work.
753 ETHAS, is a flexible and customizable tool for the ethical self-evaluation of ART procedures
754 applied to mammals in biodiversity conservation projects. The self-assessment tools help
755 scientists to be proactive and to scrutinize the ethical issues surrounding their work and make
756 them easier to be communicated, discussed, and addressed, contributing to the responsible
757 conduct of research, thereby increasing its public acceptance (European Commission, 2019). The
758 tool is based on checklists, a valuable tool for self-assessment to identify errors and check the
759 conformity to operational standards, best practices, ethical tenets (such as 3Rs), and normative.
760 The general frame of the ETHAS tool is based on two integrated checklists for self-assessment,
761 the Ethical Evaluation Sheet (EES) and the Ethical Risk Assessment (ERA). ETHAS's checklists
762 were developed with the aim of combining the risk assessment of the specific procedures with
763 the ethical acceptability assessment. The EES and ERA checklists have been developed based
764 on the current literature and best practices guidelines and refined through an iterative

765 consultation process between experts (both ethicists and scientists) and stakeholders, which is
766 still ongoing. After its development, the tool was implemented through a reiterative process
767 among the scientists of BioRescue. The tool was then applied to assess the procedure of ovum
768 pick applied to the two females still alive, currently hosted at Ol Pejeta in Kenya.
769 In the last part of section 1, it is presented the adaptation of ETHAS for the assessment of the
770 laboratory procedure used to reprogram fibroblast of cryopreserved tissues to produce iPSC and
771 the laboratory procedure to produce in-vitro gametes.
772 The laboratory procedures of aART require the manipulation of cells that may result in risks to
773 the health and welfare of future newborns and potentially to the species. This is the case when
774 ART and aART used for genetic rescue may cause genetic risks in the species, including
775 outbreeding depression, swamping of beneficial alleles, or disrupting co-adapted gene
776 complexes (Bell et al., 2019). In all these cases, it is necessary to evaluate if the level of risk is
777 acceptable or not, using the "as low as reasonable applicable principle (ALARP)" together with
778 the Precautionary Principle (Ersdal and Aven, 2008).
779 However, a risk assessment of each assisted reproduction procedure can prove if it can be
780 considered "reasonably safe" (Tickner et al., 2003). In this way, the Precautionary Principle
781 provides a certain degree of operativity for any research aiming to design new conservation
782 strategies, even if there is a certain level of unpredictability. This unpredictability can be ethically
783 acceptable only when a risk assessment is performed on the procedures to highlight potential
784 risks and evaluate them in terms of occurrence and outcome, plan mitigation actions, and
785 evaluate possible alternatives. In this way, even if the risk probability is never zero, it can be
786 taken to a tolerable threshold level. A risk assessment integrated into the ethical analysis can
787 help to evaluate a tolerable threshold level of the use of these biotechnologies in wildlife
788 conservation. The ethical analysis must also evaluate the quality of the procedures and their
789 compliance with the current legislation and the best practices in the field. Next, it must assess
790 the potential benefits deriving from safeguarding biodiversity, the possible positive social

consequences, the scientific and technological advancements that the application of these technologies can achieve, and whether they are carried out in a responsible and sustainable way. As assisted reproduction technologies procedures are performed on animal specimens, the welfare of donors and future newborns must be considered at any stage in laboratory procedures. The ethical analysis must also include an assessment of personnel safety and quality, as these aspects can be detrimental at any level for the people involved, the animals, and the valuable biomaterial collected.

A lack of attention to these facets can be detrimental to the ethical acceptability of conservation projects that apply these innovative strategies, even if their ultimate goal is commendable. ETHAS for IVF laboratory was applied to AVANTEA laboratory procedures and is part of the published paper "An ethical assessment tool (ETHAS) to evaluate the application of assisted reproductive technologies in mammals' conservation: the case of the northern white rhinoceros (*Ceratotherium simum cottoni*) " presented in section 1.2.

ETHAS for biomolecular procedures was applied to the procedures performed at the laboratory of the Department of Stem Cell Biology at the Max Delbrück Center for Molecular Medicine, Berlin, Germany, to generate iPSC from somatic tissue cells, and at the Department of Basic Medicine at Kyushu University, for in-vitro gametogenesis.

ARTs and aARTs in the future will be the most effective approach for breeding programs of endangered species to obtain stable populations in the wild.

However, for the present, to avoid their extinction, the IUCN recommends the captive maintenance and reproduction of all species whose habitat is threatened (IUCN, 2020).

These animals are often kept in zoos, conservancies, or other zoological institutions that are taking a prominent position in wildlife conservation. To be trusted in their mission, these institutions must have a positive reputation to obtain public support.

In the second section, I present independent works on the ethical analysis of new challenges in wildlife conservation.

In the first part of the section, I present the development of a tool, the zoo evaluation ethical tool (ZERS), to assess the public opinion of visitors on relevant drivers that are known to frame public opinion on corporations or institutions.

Every year, more than 700 million people, one-tenth of the world population visit zoos and aquariums every year (Bruni et al., 2008; Moss et al., 2015; Stevens & McAlister, 2003; World Association of Zoos and Aquariums, WAZA). With such vast and wide-ranging audiences, zoos can play an important role in educating children and adults on the importance of biodiversity and raising awareness of conservation challenges (Moss et al., 2015).

However, their reputation and specifically the reputation of how ethically they act is crucial to be credible. Reputation is widely studied for firms and corporations, and it is considered an intangible but highly valuable asset. Many studies have shown that corporate reputation has surpassed traditional palpable assets in determining the ability of a company to thrive because it attracts public support and more and better resources (Kaur & Singh, 2018). Still, there are few fragmented studies that focus only on certain aspects of the reputation of zoological institutions. In the present study, it is presented the design of a frame for assessing reputation, focusing on ethical aspects, of zoological institutions. To this aim, the literature on corporations was reviewed to identify the drivers that form reputation in corporations. The reputation of a zoo can be considered as the collective representation of its past actions, commitment, and ability to fulfill its mission. It represents the general esteem in which the zoo is held internally by employees and externally by its stakeholders. During this Ph.D. work. the drivers affecting the reputation of zoological institutions were identified and defined. They were dived into four categories: Functional drivers; Motivational drivers; Relational drivers; and Third-party influence drivers.

The frame was then applied to the development of a tool based on a survey, the Zoo Ethical Reputation Survey (ZERS), that could assess the opinion of zoo visitors on the specific aspect of the driver. The tool was then applied to two zoos, and the results of the study are reported in section 2.

ZERS can be used by zoological associations to evaluate how much the public perceives the commitment of their members. At the same time, the use of ZERS can also enable individual zoos to highlight critical issues and implement strategies to improve them. By addressing them, zoos can not only increase people's trust and involvement in their biodiversity conservation efforts but also, by reflecting on measurable parameters, they are encouraged to operate as ethical institutions, "ethical arks" committed to advancing higher standards and practices towards all their stakeholders.

In the second part of this last section, I present two independent works conducted to assess the consequences of COVID-19 pandemic outbreak on how online newspapers framed the news on bats, in the first study, and the perception the public had of the impact of the lockdown and other restriction periods on zoos, in the second study.

In late 2019, the first cases of atypical pneumonia of unknown origin were registered in Wuhan, Hubei, China. The aetiological agent was promptly isolated in patients' blood samples, throat swabs, and lung fluids and identified as a novel b-coronavirus of the *Coronaviridae* family (Lu et al., 2020). The International Committee on Taxonomy of Viruses (ICTV) of the World Health Organization named it "severe acute respiratory syndrome coronavirus 2", SARS-CoV-2 (ICTV Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). The disease caused by SARS-CoV-2, was named COVID-19, and on 11 March 2020, the World Health Organization (WHO) assessed that it could be characterized as a pandemic (WHO, 2020). Bats were soon suspected to be the reservoir of this new virus.

The COVID-19 outbreak offered a unique opportunity to assess communication's value in wildlife conservation globally. To this aim, the research group in which I took part gathered global media reports on bats from before and during the pandemic across 26 countries and in 7 languages to assess the content of the information of each bat-related media report and if the information contained in media reports changed throughout the first months of the COVID-19 pandemic.

Bats are mammals of the order Chiroptera with more than 1400 species of bats and represent one-fifth of the mammal species (Dutheil et al., 2021). Their genomes contain several retroviral and non-retroviral sequences that can be expressed and may have played a role in the evolution of bat immunity, creating a virus-tolerant phenotype (Skirmuntt et al., 2020). Due to their peculiar benign virus-host relationship immune system, they carry many viruses that can pass to humans through spillover (Watson, 2020). Bats have been linked to several virus families that can induce severe disease in humans, such as Rhabdoviridae, Orthomyxoviridae, Coronaviridae, and Flaviviridae (Calisher et al., 2006). However, these animals play critical ecological and economic roles as insect controllers, pollinators, and seed dispersers (Boyles et al., 2011; Kasso and Balakrishnan, 2013). Yet, bats are vulnerable to a range of human threats, ranging from well-documented habitat loss and human hunting, and close to 1000 species of bats require conservation or research attention (Frick and Kingston, 2019). Their conservation may improve ecosystem functioning, positively affecting the economy and even human health, as suggested by "One Health" approach (Deckers, 2018). Though, attitudes toward bats are largely negative (Lu et al., 2021). The connection with zoonotic diseases has considerable potential to negatively impact the human perception of bats by evoking fear and intolerance among the public (Rocha et al., 2021), especially if risk communication is poorly contextualized and inadequately crafted (Rocha et al., 2021).

Bats are often portrayed as terrifying animals. Following the so-called knowledge-deficit problem (Schultz, 2011), it can be assumed that providing people with information will result in changes in attitudes towards these animals. However, simply improving communication practices is unlikely to effectively counter misinformation or information flows influenced by certain beliefs and values (Lewandowsky et al., 2017).

Today, online newspapers give news in real-time from around the world, and their role in public information is becoming increasingly relevant. Additionally, online news can be easily shared through social media, amplifying the audience. Furthermore, as many people lack direct

experience with wildlife and form their risk perception primarily on the information provided, media play a crucial role in shaping society's attitudes toward wildlife.

The aim of our research group was to analyze the effects of the COVID-19 pandemic on how the information on bats was addressed and to assess if a biased negative representation of wildlife by the global press may undermine the conservation efforts of these animals due to possible culling or eradication. Our research showed how relevant was the impact on correct pro-conservation communication. The COVID-19 outbreak was followed by an initial outburst of news that correlate bats with viruses and diseases. However, the subsequent interventions of different conservation communication initiatives allowed pro-conservation messages to resonate across the global media, likely stemming an increase in bat persecution. Yet conservation messaging, to affect targeted behavior change or influence values and attitudes towards conservation, introduces new ethical dilemmas that should be considered (Gregg et al., 2022)

The considerations discussed among the researchers of the group during this work highlighted the relevant aspects of communication in conservation as a two-way messaging between who sends the message and who receives it and highlighted what should be done or not from both sides.

In the last part of this second section, I present the results of a survey aimed to assess the perception of the difficulties the zoological institutions faced after the COVID-19 outbreak.

The relationship between zoos and their visitor is fundamental for these institutions to be able to achieve the goals of their conservation projects. For example, research suggests that repeat visitors are more incline to support conservation efforts than those visiting zoos for the first time (Clayton et al., 2017; Godinez & Fernandez, 2019). During the lockdown and the following periods of restriction of movement of the population ordered by the Italian government to limit SARS-CoV2 transmission. Due to the fear and uncertainty of SARS-CoV2 transmission from animals to humans and vice versa, visitors could not access to zoological institutions in total or in certain areas of them (such areas hosting apes or felines) for a long period.

However, zoological institutions remained open and struggled to maintain the level of their work and animal welfare standards at the same high level they had before the pandemic. For many zoos, social media become a powerful means of communicating at a distance with people, and online fundraising became a key source of income during COVID-19 lockdowns and zoo closures (Ryder et al., 2021). However, worldwide many had to close down (Hunton et al., 2022). To assess the awareness of the Italian public on the difficulties faced by zoological institutions it was used a survey based on a questionnaire.

The questionnaire administration was done using a simple, quick, and anonymous online survey tool, LimeSurvey, and the link was disseminated via online media or in the presence of zoo visitors by researchers. The results showed the public was aware of the economic difficulties of zoos during the pandemic and that scientific research and educational programs had a negative impact.

According to the One Health approach, the loss of biodiversity and the human exploitation of wildlife will cause new virus spillovers (Buttke et al., 2015). Scientists are working to systematically evaluate novel wildlife-origin viruses in terms of their zoonotic spillover and spread potential (Grange et al., 2021). Zoological institutions should also start to think about how to manage a "continuum of pandemic phases" (WHO, 2017) from the point of view of "safety concepts" (Lindhout & Reniers, 2020). Assessing the public awareness of the impact of the COVID-19 pandemic on zoological institutions is relevant because it can help to understand what can be done to better engage the public in case of similar situations.

During my Ph.D., in all the works here presented, I applied the principles of conservation ethics of applied ethical tenets to analyze ethical issues in conservation projects and to develop ethical tools, the principle of an ethics reputation for assessing the reputation of zoos, and the principle of ethical communication to assess how bats new were framed after COVID-19 outbreak.

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SECTION 1

ETHICAL ANALYSIS OF INNOVATIVE APPROACHES IN WILDLIFE CONSERVATION

1 .1 Ethical Analysis of the Application of Assisted Reproduction Technologies in Biodiversity Conservation and the Case of White Rhinoceros (Ceratotherium simum) Ovum Pick-Up Procedures.

This part of my Ph.D. has been developed as member of the ethical team of the international BioRescue consortium, and the following chapter is an adaptation of:

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Abstract

Originally applied on domestic and lab animals, assisted reproduction technologies (ARTs) have also found application in conservation breeding programs, where they can make the genetic management of populations more efficient, and increase the number of individuals per generation. However, their application in wildlife conservation opens up new ethical scenarios that have not yet been fully explored. This study presents a frame for the ethical analysis of the application of ART procedures in conservation based on the Ethical Matrix (EM), and discusses a specific case study—ovum pick-up (OPU) procedures performed in the current conservation efforts for the northern white rhinoceros (*Ceratotherium simum cottoni*)—providing a template for the assessment of ART procedures in projects involving other endangered species.

Introduction

Assisted reproduction technologies (ARTs) offer increasingly important opportunities for biodiversity conservation (1–3). Originally applied mainly on domestic and lab animals, ARTs have found usage also in conservation breeding programs, where they can enhance the genetic management of populations, and increase the number of offspring per generation. More elaborate and costly techniques, advanced assisted reproduction technologies (aARTs) not commonly employed on livestock and laboratory animals, may even spark hope for the survival of taxa that are functionally extinct or at the verge of extinction (4, 5).

However, the application of ARTs in biodiversity conservation opens up new ethical scenarios that have not yet been fully explored. Like any other technology capable of redefining the boundaries of extinction (6), ARTs question the very idea of conservation we want to pursue and the values it needs to convey. Moreover, ART applications may have ethically relevant consequences—on conservation projects, on the people involved or otherwise affected, and on the animals on which they are performed—that should be carefully discussed.

The ethical assessment of the involved procedures is an integral and crucial part of the ethical assessment of conservation projects (7). Here, we propose a frame for the ethical analysis of ART procedures in conservation using the Ethical Matrix (EM), and we discuss a case study based on ovum pick-ups (OPUs) performed for the current conservation efforts of the northern white rhinoceros (NWR, *Ceratotherium simum cottoni*, Lydekker, 1908).

The NWR is a subspecies of the white rhino (*Ceratotherium simum*, Burchell, 1817) of which only two females remain (8), and whose fate is irremediably tied to the recovery and manipulation of the existing biomaterials. It should be noted, however, that the entire Rhinocerotidae family, consisting of five extant species— white rhinoceros, black rhinoceros (*Diceros bicornis*, Linnaeus, 1758), Sumatran rhinoceros (*Dicerorhinus sumatrensis*, Fischer, 1814), Javan rhinoceros (*Rhinoceros sondaicus*, Desmarest, 1822), and the greater one-horned rhinoceros (*Rhinoceros unicornis*, Linnaeus, 1758)—is currently under severe threat due to habitat loss and persistent poaching (9). In particular, black, Sumatran, and Javan rhinoceros are

critically endangered—with the latter two species reduced to small (>80 individuals and 46–66 individuals, respectively) dwindling populations (10,11). Moreover, even the less endangered taxon—the southern white rhinoceros (SWR, *Ceratotherium simum* simum, Burchell, 1817)—while "only" near threatened in the wild (12), does not have self-sustainable captive populations (13). It is likely that, among other strategies, future conservation efforts of rhinoceros will resort to ARTs (5). While new technologies like stem cell- associated techniques and *in vitro* follicular growth (5) may eventually ensure a stable supply of gametes without the need for *in vivo* collection, in the near future, procedures like OPU and semen collection will presumably remain the only viable methods to obtain the necessary biomaterial for *in vitro* embryo production. It is necessary, then, to analyze the ethical issues associated with these interventions.

The purpose of this study is, therefore, three-fold: (i) to provide a methodology for the ethical analysis of ART procedures in conservation projects; (ii) to use this methodology to assess the OPU procedures performed in the case study; (iii) to provide a template for the assessment of OPU procedures in other projects involving white rhinoceros or other members of the Rhinocerotidae family.

Materials and methods

Assessing ARTs in Conservation Projects

In human medicine, ARTs are usually defined as those procedures or treatments in which both the male and female gametes or embryos are manipulated *in vitro* to achieve pregnancy (14). In contrast, in veterinary medicine, the catalog of ART is normally broader, including, for instance, artificial insemination (15–21), cloning via somatic cell nuclear transfer (3, 22–25), and gamete production from induced pluripotent stem cells (3, 5). Following this broader use, the term ART will hereinafter be applied to any procedure involving, in one or more of its stages, the manipulation of reproductive cycles, gametes, or embryos with the final aim of producing a new

individual.

With biodiversity conservation, we mean, instead, those scientifically grounded activities aimed at managing natural environments, ecosystems, wildlife, flora, biotic process, and, more generally, the whole biosphere with the end of maintaining and, eventually, restoring, the natural diversity of life on our planet and its evolution processes at all biological levels—from the ecosystem to genes. Biodiversity conservation is an ethically significant activity since it preserves the source of different kinds of values, both instrumental and non-instrumental.

Applications of ARTs in livestock, laboratory animals, and wildlife usually differ in their goals. In livestock and laboratory animals, ARTs are primarily used to maximize the offspring from genetically desired individuals. Producing large numbers of individuals with certain recurring genetic characteristics is instead generally neither useful nor desirable in the context of wildlife conservation. Rather, the goal of what could be termed "conservation ARTs" is to assist in the establishment of self- sustaining populations for reintroduction or as a genetic reserve. ARTs can contribute to this goal in two complementary ways. They can help increase the number of individuals in each generation, by expanding the opportunities and chances for achieving pregnancy. Moreover, they can improve the genetic management, by facilitating the breeding between spatially separate animals without the need for translocation, and by reintroducing into the gene pool those individuals who, for various reasons, are incapable of mating or breeding—including dead individuals whose suitable biomaterials have been cryopreserved.

Ethical analysis is crucial when conservation ARTs are involved. ART procedures in wildlife, for instance, are usually less established and—in some cases—more demanding for the subjected animals than those performed on the domestic animals. Moreover, given the different goals, some of the techniques used in conservation are more complex, as well as more challenging in terms of equipment and veterinary expertise required, than those normally employed for livestock. Finally, by redrawing the boundaries of the concept of reproduction—and, in some cases, of extinction—conservation ARTs can have a social and scientific impact that must be scrupulously considered.

The Frame for the Ethical Analysis of Conservation ARTs

Ethical analysis permits us to determine whether a procedure is acceptable according to certain standards of value and to identify the critical issues that need to be addressed before its implementation. This should not be confused with the assessment of the project, or with the assessment of the specific implementations of the procedure. In the first case, the focus is much broader. In the second case, there is the need to include the various contextual variables in the evaluation. In both cases, however, the ethical analysis of the procedures provides a fundamental support: as an essential part of project assessment, and as a backbone for the assessment of implementations.

Carrying out a comprehensive ethical analysis of a specific conservation ART procedure means identifying and gathering numerous relevant factors beyond the technical and scientific details of its execution. The procedure has to be considered in the context of the project it is part of, and in the broader perspective of biodiversity conservation. Moreover, as conservation activities take place at the crossroad between different value dimensions (26), the procedure has to be evaluated in its wider effects on animals and people, that is, beyond its mere conservation value.

The factors to be considered for conducting a thoughtful ethical analysis of conservation ARTs can be grouped into five categories. One category revolves around the immediate context of the procedure, that is, around the project it belongs to, its goals, the probability of achieving them, and the values they convey. Some questions to be raised in this regard are as follows: What are the goals of the project? Have success criteria been clearly defined? How reasonable are the chances of success of the project according to these criteria? What is the conservation value of the project? What other values are brought forward by the project? In case of failure, would the project still lead to some kind of valuable advancement (ecological, scientific, social, etc.)? An exhaustive answer to the above questions would require a detailed analysis of the overall project and is therefore not feasible when assessing a procedure. However, it is still necessary to have a sufficiently defined picture of the ultimate reasons why the procedure is undertaken, as this provides the context for assessing eventual critical aspects.

Moreover, it is necessary to focus on the role of the procedure in the project and its effectiveness in reaching the assigned goals. What purpose does the procedure serve in the project? Is the success of the procedure a key part of the project? Can there be alternatives in case of failure? Is it the most effective way to perform the task assigned? Have the alternatives been considered? How has the procedure been chosen? Besides the reasons for efficiency, the effectiveness of a procedure is a central issue where ethically relevant risks or costs are present. Moreover, the reasons that led to the inclusion of the procedure into the project should also be made explicit and examined to detect eventual biases.

The procedure must also be analyzed beyond its immediate contribution to the project. This means investigating its possible value beyond its effectiveness in carrying out the specific goal of the project. For instance, what is the scientific value of performing the procedure? Can it lead to scientific and technological improvements? Does it establish or refine protocols that could be employed in other biodiversity conservation projects? Can carrying out the procedure have a positive impact on the welfare of the animals involved? Can it have a positive social effect of some kind, for example, by promoting knowledge transfer or capacity building? While procedures do not happen in a vacuum, meaning that their implementation always happens in a project, the project itself may not exhaust their usefulness. Answering the above questions permits us to extend our understanding of the possible merits of the procedure beyond its instrumental value for the project.

Special attention should also be paid to the risks and costs associated with the procedure itself. What are the known risks of performing the procedure? Who is responsible? Can the procedure harm the welfare of the animals involved? Does it put at risk their lives? Are there risks for people? What could be the repercussions in case of failure? Are there any negative side effects to consider in case of success? As veterinary interventions, conservation ARTs invariably entail some risks during their performance as well as before and after (translocation, handling, restraining, recovery, etc.). These risks should be investigated and their distribution among the different involved stakeholders should be made clear, since this, alongside the distribution of

benefits, is important to evaluate the acceptability of the procedure.

The last category of ethically relevant factors focuses on how the procedure fits into the values and worldview of public opinion and conservationists. Does the procedure raise public concerns? Are there any groups that particularly oppose it? Why? How does the procedure match or challenge the various existing perspectives on biodiversity conservation? Public opinion can be skeptical of the project and the employed procedures. Sometimes this is just due to lack of involvement or inadequate information. However, in other cases, the reasons can be more substantial: the unfair distribution of the costs and benefits of the project among the people and communities involved; there is distrust for the individuals or the institutions carrying out the project; the goals and the methods of the project conflict with the shared values, etc. Similarly, uses of conservation ARTs may challenge the tenets of some conservation philosophies. A careful analysis of the factors in this category allows for the anticipation of potential conflicts so that it should be possible to take countermeasures.

Gathering Factors Through the EM

Table 1 summarizes the necessary factors to be considered for analyzing the applications of conservation ARTs. Some factors (i.e., the goals of the project, feasibility, and the effectiveness of the procedure) can be retrieved from the description of the project itself. Other factors must instead be identified by analyzing the procedure from an ethical standpoint. To achieve this goal, a specific ethical tool—the EM—can be applied.

Table 1. Relevant factors for the ethical analysis of conservation ARTs.

Group	Factors to be investigated	Examples of associated questions
• Context of the procedure	• Goals of the project • Values conveyed by the project's goals • Feasibility of the project	• What are the goals of the project? • Have success criteria been clearly defined? • How reasonable are the chances of success of the project according to these criteria? • What is the conservation value of the project? • What other values are brought forward by the project? • In case of failure, would the project still lead to some kind of advancement (ecological, scientific, social, etc.)?
• Role of the procedure in the project	• Value of the procedure for the project • Effectiveness	• What purpose does the procedure serve in the project? • Is the success of the procedure a key part of the project? • Can there be alternatives in case of failure of the procedure? • Is it the most effective way to perform the task assigned? • Have alternatives been considered? • How has the procedure been chosen?
• Value of the procedure beyond the project	• Scientific value • Conservation value • Animal welfare value • Social value	• What is the scientific value of performing the procedure? • Can it lead to scientific and technological improvements? • Does it establish or refine protocols that could be employed in other biodiversity conservation projects?
• Risks and costs of the procedure	• Known risks of the procedure, and their distribution Costs of failure of the procedure • Negative side-effects of the procedure in case of success	• Can carrying out the procedure have a positive impact on the welfare of the animals involved? • Can it have a positive social effect of some kind, for example by promoting knowledge transfer or capacity building? • What are the known risks of performing the procedure? • On who do they fall? • Can the procedure harm the welfare of the animals involved? • Does it put at risk their lives? • Are there risks for people? • What could be the repercussion in case of failure? • Are there any negative side-effects to consider in case of success?
• Views on the procedure	• Public opinion's views on the procedure • Conservationists' views on the procedure	• Does the procedure raise public concerns? • Are there any groups that particularly oppose it? • Why'? • How does the procedure match or challenge the various existing perspectives on biodiversity conservation?

The EM permits us to unpack and analyze the ethically relevant aspects involved in a complex scenario, reorganizing them into a transparent and comprehensible picture of value demands. Originally developed by Mepham (27) for the ethical assessment of technologies and policies in agriculture and food processing, the EM has since been applied in many other fields—including veterinary medicine (28, 29), forestry (30), aquaculture (31, 32), assessment of human–animal interactions (33, 34), management of contaminated agricultural ecosystems and radioactive waste (35, 36), and conservation (37).

The EM embraces a pluralistic ethical approach. Cells from the first column of the EM list stakeholders. Cells from the first row list three general ethical principles, influential, recognized, and shared tenets of ethical reasoning and common morality such as wellbeing, autonomy, and fairness (38, 39). Intersecting cells list the value demands for the stakeholders derived from the general ethical principles.

The EM specifically tailored for conservation (40) includes three categories of potential stakeholders: ecological entities, individual animals, and people. Table 2 recaps the general value demands generated by applying the ethical principles on these categories of stakeholders.

The methodology of the EM is to apply the general template on a specific case, first by identifying the stakeholders involved, and then by applying the general ethical principles in order to derive the value demands.

Table 2. General EM.

	Wellbeing	Autonomy	Fairness
Ecological entities	Conservation	Freedom from human intervention	Equal treatment in relation to conservation
Animals	Health and functioning Absence of negative affective states and allowance of positive ones	Living natural lives and expressing species-specific behaviors	Equal treatment in relation to welfare
People	Psychological and physiological welfare Sustainable social, economical, and cultural welfare	Freedom of choice Capacity to exercise the various fundamental aspects of one's own persona Self-determination	Equal and fair treatment

The Case Study: OPU on White Rhinoceros

As a case study, we analyzed the OPU procedures performed in the recent conservation efforts to save the NWR. The case appears interesting due to several reasons. It is rather complex, with many ethically relevant issues packed together; it involves many stakeholders and multiple value dimensions, with a variety of potential value conflicts; the ART techniques employed in the project have the potential to redefine the boundaries of wildlife reproduction and extinction.

The most peculiar aspect of the case is that the NWR has been declared "functionally extinct" (8). From ~2,230 individuals in 1960 (41), the wild population of NWR has been reduced, mainly by poaching, to a few individuals by the 1980s, and presumably erased sometime after 2007 (8). During the same years, the small population kept in zoos proved to be not self-sustainable. White rhinoceros have a low reproductive rate in captivity (42). Despite various

breeding attempts, only four NWR offspring were ever known to be born in this way (at the Dvu˚r Králové Zoo). Since the death of the last male Sudan in 2018, two females, both living at Ol Pejeta Conservancy in Kenya, have become the lasts of their kind. They are Najin, aged 32, and her offspring Fatu, aged 21.

The current conservation efforts for the NWR by the BioRescue project—an international consortium coordinated by the Leibniz Institute for Zoo and Wildlife Research in Berlin—combine aARTs and stem cell-associated techniques (43). Frozen semen from five NWR males is available, and the stored tissue could be used in the future to produce gametes by using novel technologies. Due to severe reproductive pathologies, both the remaining females cannot carry to term a pregnancy. In the case of the older female, this is due to tendon problems in the hind legs; in the case of the younger, this is due to the uterine pathology of an unknown origin. The only current way to "de- doom" the taxon is to collect their oocytes to create embryos using intracytoplasmic sperm injection (ICSI) to be transferred into SWR recipient cows.

The first point to be made here is that, despite the possible similarities, this conservation effort must not be confused with an attempt at de-extinction. De-extinction can be defined as the process of bringing back an extinct taxon (6), and it can be divided into two categories: the de-extinction of recently extinct taxa, and the de-extinction of species that had gone extinct hundreds or thousands of years ago, and whose significant ecological relationships have now disappeared ["deep de-extinction"; (44)]. Both the categories raise specific conceptual and ethical challenges (45). While de-dooming a functionally extinct taxon like the NWR may resemble a case of de-extinction in the first, non-deep, sense—in both scenarios the original ecological context still exists—the two differ in a decisive aspect, that is, generational continuity. Generational continuity cannot be recreated through de-extinction, and this may constitute in some taxa both an ecological and ethical issue: ecological, as some behaviors and functions can only be acquired through interaction between adults and juveniles; and ethical, as animal welfare may be harmed by the absence of these behaviors and functions. For these reasons, it makes sense to keep conceptually distinct the actions of de-dooming a functionally extinct taxon and de-extinguishing

a vanished taxon. In the case of NWR, since only two females remain, one of which had no offspring—the generational continuity is at least partially impoverished. Nevertheless, it has not disappeared altogether, and SWR individuals can be used as proxies for NWR adults to transmit those behaviors that are known to be similar between the two subspecies, such as reproductive behaviors (46).

In vivo oocyte collection in rhinoceros is a relatively new intervention. The full procedure as it is performed currently in white rhinoceros involves ovarian super stimulation, full anesthesia, and transrectal ultrasound-guided oocyte recovery (17, 43, 47). In the addressed context, the procedure has been conducted regularly (albeit with at least 3 months of lapse) in the two remaining NWR females (48). Table 3 recaps the results of the seven procedures that have been executed so far in NWR. Overall, the procedure has been rather successful in Fatu, with 95 oocytes collected in seven OPUs between 2019 and 2021, which have been used to produce a total of 13 embryos. The procedure has been less successful with Najin presumably due to her age and health, and the partners in the project have decided to discontinue performing OPU on her in 2021. Although this choice further reduces the gene pool available for embryo creation, it was preferred over other options after carefully considering the ethical and scientific elements involved (49).

At the same time, SWR oocytes are also collected from females across European zoos, in order to establish the technology also for this taxon and to synergistically support the research related to the project.

Table 3. Results of OPU and ICSI on NWR.

	Najin		Fatu	
	Oocytes	**Embryos**	**Oocytes**	**Embryos**
1. (08/22/2019)	5	0	5	2
2. (12/17/2019)	3	0	6	1
3. (08/18/2020)	2	0	9	0
4. (12/13/2020)	0	-	14	2
5. (03/28/2021)	-	-	21	4
6. (07/06/2021)	-	-	17	3
7. (10/25/2021)	-	-	23	1
Total:	10	0	95	13

Results

Building Up the EM

Following the proposed methodology, an EM for the procedure has been developed (Table 4) using the template provided in Table 2. The stakeholders included in the EM are biodiversity, the individual females subjected to the procedure, and all people involved in the project.

The level of resolution of the EM could be increased by adding more stakeholders or breaking down the existing ones into more specific items. It could be possible, for instance, to break down biodiversity into the different rhino species and the ecosystems involved or to add to the list the NWR calves born as a result of the project, the conservationist community, etc. Such a high-resolution EM would be especially useful to analyze the whole project in detail. However, since the goal is to assess a specific procedure, increasing the resolution of the EM is neither necessary nor desirable.

1663 **Table 4.** EM for OPU in NWR conservation efforts.

	Wellbeing	Autonomy	Fairness
Biodiversity	Conservation • NWR has a historical-naturalistic value. • NWR has an ecological value. • Cryobanking is a good conservation strategy per se (collect now or regret later). • Refining through application the OPU procedure may open new ways for the conservation of other taxa. • Incidents or complications during the procedure could damage the image of the project and of conservation ARTs in general.	Freedom from human intervention • Conservation ART may be deemed a technofix. • Conservation ART may lead to moral hazard. • Conservation ART may be deemed hubristic	Equal treatment in relation to conservation • Charismatic animals like rhinoceros receive a disproportionate amount of attention. • However, conservation of the NWR could benefit the conservation of other less charismatic species. • The opportunity costs of the project do not fall on more traditional conservation efforts, including conservation of other rhino taxa.
Rhino females subjected to the procedure	Health and functioning. Absence of negative affective states and allowance of positive ones • Some aspects of the procedure may harm the animals according to these dimensions of welfare. More specifically: ovarian superstimulation, anesthesia, transrectal puncture all bear a	Living natural lives and species-specific behaviors • The procedure increases the possibility for some of the animals involved to express social behaviors currently not accessible.	Equal treatment in relation to welfare • The animals involved are treated like a means for the conservation of their taxon. • However, they receive extra veterinary screening and care.

	possible risk of side-effects.		
People	Psychological and physiological welfare • Affective value for people caring for the animals. Sustainable social, economical, and cultural welfare • Economic value of the animals. • Ecotourism.	Capacity to exercise the various fundamental aspects of one's own persona • The procedure is an opportunity for professional growth, knowledge transfer, and capacity building. • NWR may have eudaimonistic (aesthetic, scientific, and reverential) value for people. • NWR may have transformative value for people. • NWR may have existential value for people.	Equal and fair treatment • Costs and benefits of the procedure should be distributed equally, and compensation given whenever this is not possible.

Biodiversity

The three basic value demands for biodiversity are (refer to Table 2): (i) conservation (under wellbeing); (ii) freedom from human intervention (under autonomy); (iii) and equal treatment in relation to conservation, without bias grounded on human preferences (under fairness).

From the standpoint of conservation, at least three values can be attached to the goals of the project, that is, bringing the NWR population back to a viable level—attaining demographic security and stability (50)—and subsequently reintroducing the taxon into the wild. The first two values are the historical and the naturalistic values of the subspecies—being a unique and

irreplaceable product of the evolutionary process which would be lost for purely anthropogenic reasons. The third value is the ecological value of this taxon. Mega-herbivores are important ecosystem engineers whose contribution to shaping their environment cannot be replicated by smaller herbivores (51). White rhinoceros make no exception to this rule, and their presence can make a difference in preserving the African savannah ecosystem (52, 53). Reintroducing the NWR would then be a way to restore and maintain the ecological relationships that are now lost. The OPU procedure has also an additional conservation value which is independent from the succes or the failure of the project. Due to the mounting extinction crisis (54), cryobanking biomaterial from endangered taxa has become an important conservation goal (19, 55), following the imperative to collect now, or regret later (5). Moreover, by carrying out the procedure, it is possible to collect technical and scientific data for developing OPU protocols in other rhino taxa, or even in other large mammals, expanding in this way the opportunities for their conservation. However, accidents during the procedure could damage the image of the project.

From the standpoint of freedom from human intervention, this procedure, like other conservation ARTs, could be considered a negative example of "technofix" that is, the use of a technology to reverse the outcomes of morally problematic activities (in this case, poaching and habitat loss) leaving intact the causes (56). Similarly, the methodology of the project could be accused of making wildlife decline overly mundane, by providing, at least in theory, an "easy" way to revert the phenomenon. This could create a moral hazard, which, in turn, could help further accelerate the extinction crisis. Finally, applications of conservation ARTs to de-doom the functionally extinct taxa may be accused to be an aggressive form of conservation, through which we attempt to forcefully impose our scheme and solutions on reality, following a hubristic attitude which has already been shown to be a part of the problem and not of the solution.

Considering equal treatment in relation to conservation, the question may be raised as to why concentrate so much effort and resources on one rhino subspecies when there are so many other endangered taxa. Rhinoceros are among the most charismatic animals (57), and this may be an explanation, albeit one that clearly expounds a bias. However, there are good reasons for not

considering the choice of the NWR as unfair. Rhinoceros can serve as umbrella and flagship species (58), meaning that the reintroduction of the NWR could foster the conservation of other less charismatic species (59). Furthermore, as previously mentioned, the refinement of conservation ART protocols could open new opportunities for the conservation of other rhino taxa or even other large mammals.

In fact, one of the advantages of this project is that its opportunity costs do not fall on other more traditional conservation endeavors, including other rhino conservation efforts. This is because it draws on funds allocated for biotechnology, and does not make use of the money collected for conservation of other rhino taxa.

Females Subjected to the Procedure

Table 2 lists three basic value demands for the females subjected to the procedure: (i) health and functioning and absence of negative affective states and allowance of positive ones (under wellbeing); (ii) living natural lives and species-specific behaviors (under autonomy); (iii) equal treatment in relation to welfare (under fairness). This captures the multidimensional nature of animal welfare (60) and should help in gathering useful elements for the assessment relative to the risks and costs of the procedure and its value beyond the goals of the project.

Regarding the first value demand, OPU on rhinoceros is a relatively new intervention, and, as such, there is no specific and systematic investigation of its effects, immediate or prolonged, on any of the previously defined criteria of animal welfare. An overall evaluation can nevertheless be attempted, starting with some considerations to be extrapolated from similar (yet not analogous) interventions performed on other species. OPUs have been performed regularly on domestic animals in the recent decades. *In vivo* oocyte collection was first performed on cattle via laparoscopy (61), and, a few years later, transvaginal ultrasound-guided follicle aspiration was introduced (62, 63). Today, laparoscopic OPU is still used in small ruminants, such as sheep and goats (64), while transvaginal ultrasound-guided OPU has become the standard for cattle, buffalo, and horses (65, 66). Applications of these methods to exotic species were first performed

in the mid-nineties (67), starting with zebras (68), and llamas (69).

Transvaginal ultrasound-guided OPU procedures are regularly repeated in the same cattle and buffalo cows twice per week (66, 70–72), as this is the frequency that assures the best yield of the oocytes (65). Horses can be subjected to OPU procedures on a biweekly schedule (73). The effects of the procedure and of its steady repetition in cattle, buffalo, and horses concerning the reproductive and productive capacities of the treated animals are well-documented (65, 70, 74–76).

In this regard, there is a general consensus that OPU procedures, even when reiterated regularly and for prolonged periods of time, do not have particularly adverse side effects. Studies with a stronger focus on criteria relative to the minimization of unpleasant affective states, partly caution this optimism, highlighting some invasive aspects of the OPU procedure. While repeated transvaginal punctures seem not to provoke the signs of short- and long-term stress neither in cattle (77), nor in buffalo cows (78), other possible sources of welfare impairments are nevertheless present, namely the possibility of minor ovarian alteration, and, most importantly, the negative physiological and behavioral responses to the epidural anesthesia administered during the procedure (79, 80). Studies on the reaction of horses to transvaginal ultrasound-guided OPU in terms of pain and discomfort are few and less systematic (24), but possible negative side-effects of the procedure have been reported (81–83).

In general, the OPU procedure on rhinoceros is related to those practiced on horses and cattle (43, 47). Horses, in particular, being members of the order *Perissodactyla* like rhinoceros, are considered good models due to their taxonomic relatedness. However, two crucial differences between the specific procedures complicate any possible linear comparison: the transrectal instead of transvaginal approach, and the full anesthesia.

The length of the reproductive tract, and the impossibility of palpating the ovaries through the rectum, make the transvaginal approach unfeasible in rhinoceros (except for the Sumatran rhinoceros). Since the classic laparoscopic approach is equally unfeasible (47), OPU in rhinoceros is performed transrectally (84). This raises issues of limited sterility of the procedure

and of the possibilities of infection. Indeed, even if restricted to a single penetration of the rectal wall, OPU in rhinoceros still poses a minimal risk of bacterial contamination of the puncture needle even after a prior thorough cleaning and disinfection of the rectum (47).

Moreover, safe immobilization and full anesthesia are required to perform the OPU procedure in rhinoceros. Full anesthesia prevents unexpected movement, limiting the risk of injuries both to the animal and to the people carrying out the operation, yet it poses its relevant risks of complications. Standard anesthesia protocols in rhinoceros are etorphine hydrochloride-based (85). Some of these protocols have been reported to be suitable for weekly (86) and bimonthly (87) anesthetization of the same animals—a black rhinoceros and a greater one-horned rhinoceros, respectively. Nevertheless, anesthesia in general, and the use of etorphine-based protocols in particular, have been associated with many potential and possibly fatal complications, including aspiration, respiratory depression, hypoxemia, hypertension, pulmonary shunting, and ventilation/perfusion mismatch (88–91). Moreover, etorphine can be very dangerous to people, and cases of accidental exposure, while very rare, are reported in the literature (92, 93).

Transrectal oocyte retrieval is preceded by ovarian stimulation. The ovarian stimulation protocols administered to the animals employ Histrelin, a slow-release GnRH analog. The GnRH analog is injected every other day either three or four times before the OPU procedure. Captive white rhinoceros are known to suffer from various genital tract pathologies, most likely favored by long non-reproductive periods (94). Hormonal stimulation could potentially contribute to the progression of these pathologies.

From the standpoint of the second value demand, that is, the possibility of living natural lives and expressing species-specific behaviors, the procedure, by contributing to the success of the project, could be evaluated positively, at least for the two NWR females, as it may provide them, in the medium term, with a chance for expressing some parts of their behavioral repertoire which are currently not accessible. White rhinos form cow-calf and cow-adolescent pairs, which are typical groupings in the social structure of the species, with no need for males to rearing a calf

(95). This means that there is a concrete possibility that the remaining females could establish social bonds with the newborn NWR. In this regard, it is important to note that, although both Najin and Fatu were born in captivity, they were accompanied during their earlier lives by several other captive-born as well as wild-caught NWR, and had, in this way, enough opportunity to learn social behaviors from conspecifics. Although it is not possible to determine a priori to what degree the normal social structure of the species can be recovered from this bottleneck of two individuals, returning the population to viable numbers could allow its members to cultivate a wider range of species- specific social behaviors.

On the other hand, from the standpoint of the third value

demand, equity regarding welfare would require managing similar animals in the same manner. This is violated as soon as the animal is subjected to a procedure that could cause stress, discomfort, and even, in the worst cases, harm, without any direct and substantial benefit. However, while it is undeniable that in the procedure animals are mainly treated as a means for a goal—the collection of oocytes—which is only tangentially tied to their wellbeing, it is equally true that they receive much more veterinary screening and care than what constitutes the norm for white rhinoceros in captivity. Given the particular vulnerability of captive female rhinoceros to reproductive tract pathologies, such as tumors (42, 94, 96, 97), this is not an aspect to consider lightly.

People Involved in the Project

Table 2 lists three basic value demands for people involved in the project: (i) psychological and physiological welfare and sustainable social, economical, and cultural welfare (under wellbeing); (ii) freedom of choice, capability to exercise the various crucial aspects of one's own persona, as well as self- determination (under autonomy); and (iii) equal and fair treatment (under fairness). This should help in gathering useful factors for the assessment relative to the context of the procedure, of its value beyond the project and of its risks and costs.

Considering the first value demand, it is important to note that several people—keepers, veterinarians, caregivers—have regular, if not daily, contact with the animals involved, and may have built affective bonds with them. It may be expected that these people will be especially concerned for the safety of the animals during the procedure.

A second aspect to note is that the animals involved have a certain economic value, which could be reduced in case of complications during the procedure. At the same time, communities living in the area of the eventual reintroduction of the NWR could benefit from the success of the project, as it could create new opportunities for ecotourism.

Concerning the second value demand, the possibility of performing the procedure can be both an opportunity for professional growth and, given the international nature and the cutting-edge technologies of the project, an occasion for knowledge exchange and transfer. Re-establishing a self-sufficient population of NWR and reintroducing it could also promote several kinds of values linked to our fulfillment as individuals (98, 99). Indeed, majestic animals like rhinoceros can be sources of aesthetic value, scientific value, reverential value, and transformative value—meaning with this latter, the capacity of producing powerful and even life-changing experiences. Moreover, even just knowing that the NWR has been saved from extinction can be important for many people (the so-called existential value of biodiversity), even if they cannot directly experience or benefit from this.

Concerning the third value demand, a requirement should be that costs and benefits of the procedure be distributed equally, and compensation should be given whenever this is not possible.

Discussion

Factors for the Assessment

Along with the results from the project description, the value demands listed in the EM can be used to gather the factors for the ethical analysis frame presented before. Table 5 shows the outcome of this process.

1834 **Table 5.** Factors for the ethical analysis of OPU procedures in white rhinoceros.

Category	Factors to be investigated	Description
1. Context of the procedure	• Goals of the project • Values conveyed by the goals • Feasibility	• The ultimate goal is to create a self-sustaining population of NWR to be reintroduced into the wild. This will be the ultimate criterion of success of the project. • Such a goal conveys several form of value: - Historical, naturalistic and ecological value directly tied to saving the NWR from extinction and reintroducing it. - Welfare value, for giving to the two remaining NWR the chance to exercise social behaviors currently not accessible. - Economic value, tied to the opportunity for ecotourism. - Transformative value for people, as encounter with NWR could lead to life-changing experiences. - Eudaimonistic (aesthetic, scientific and reverential) value, as encounter with NWR could lead to significative experiences. - Existential value, as people could still find valuable the existence of the NWR even without directly experiencing it. • It is not possible to establish with absolute certainty that the project is inevitably destined to succeed due to the limited access to biomaterial and the cutting-edge technology it requires. • The scientific and conservation values fulfilled by the refinement of protocols could still be realized even in case of failure of the project.
2. Role of the procedure in the project	• Value of the procedure for the project • Effectiveness	• Performing the OPU procedure is needed to collect the necessary oocytes for refining the ICSI and ET protocols, defining embryo quality standards, and creating NWR embryos. For this reasons, it is a key part of the project. • While gamete production from somatic cell associated-techniques can perform a crucial

		complementary role to the OPU procedure, techniques are still in the process of being adapted to rhinoceros. • The OPU procedure has shown to be rather effective, with 95 oocytes retrieved so far from a single NWR female, Fatu, in 7 interventions, and 13 embryos created via ICSI (see Table 3).
3. Value of the procedure beyond the project	• Scientific value • Conservation value • Animal welfare value • Social value	• Beyond its instrumental value for the project, the procedure conveys several other forms of value: - Scientific and conservation values for cryopreserving biomaterial from an endangered taxon and refining new protocols that could be used for projects involving other taxa. - Welfare value, as extra veterinary screening and care is provided to the animals involved. - Social value, by fostering knowledge transfer and the development and strengthening of links between people, groups and institutions dedicated to conservation.
4. Risks and costs of the procedure	• Known risks, and their distribution • Costs of failure • Negative side-effects in case of success	• Some parts of the procedure (ovarian superstimulation, anesthesia, transrectal ovarian puncture) may lead to complications that could harm the animals involved. • Negative repercussions in case of complication could be: animal welfare impairment; economic damage to the owners; suffering to people who had established bonds with the animal; damage to the image for the project and for the entire conservation world.
5. Views on the procedure	• Public opinion's views on the procedure • Conservationists' views on the procedure	• Conservation ARTs may be accused of being a technofix, of creating a moral hazard, and of being hubristic.

Context of the Procedure

The ultimate goal of this conservation effort is to create a self- sustaining population of NWR to be reintroduced into its still existing natural habitat. Establishing a population with these

characteristics is, therefore, the ultimate success criterion of the project. This goal conveys many kinds of values: from the historical and naturalistic to the ecological, economic, transformative, eudaimonic, and existential. Success would also provide for some of the involved females to expand their current range of accessible social behaviors.

To reach this goal, the development of technologies and protocols, not yet available (at least for rhinoceros), is required. This means that it is not possible to establish with absolute certainty that the process is inevitably destined to succeed. However, some of the values conveyed by the project would still be fulfilled even in the event of a failure. Given its use of cutting-edge technologies, for instance, the scientific value of the project will still be high even in case of failure and the accumulated knowledge could be used to establish and improve similar procedures. Moreover, there are no opportunity costs falling on traditional conservation efforts, because the project draws from funds allocated for biotechnology and does not use the money raised for the purpose of funding conservation of other rhino taxa.

However, even in the case of success, some aspects must be taken into account when providing an overall evaluation of the project. One aspect concerns the welfare of the newborn calves. Although there is no reason to think that the calves will receive less attention than other white rhinos born in captivity or residing at Ol Pejeta Conservancy, it is not possible to know, in advance, if social interaction problems may arise due to rearing issues. A second aspect to be taken into consideration concerns the possible reintroduction of the NWR into the wild. In addition to all the welfare issues that can arise during a reintroduction (100), the chances of success for the operation lie on the possibility of removing the causes that led in the first instance to the decimation of this taxon, which have to be traced primarily in poaching.

Role of the Procedure in the Project

The OPU procedure is a key part of the project. In the SWR females, OPUs are performed to obtain the biomaterial needed for establishing new protocols for *in vitro* embryo production via ICSI and embryo transfer (ET). This is fundamental both for the “de-dooming” of the NWR as

well as for establishing self-sustaining captive backup populations of SWR and helping with their future conservation. In the NWR females, OPUs are performed for producing embryos to be implanted as soon as the protocols for ET are ready. Presently, no alternative exists to this method of obtaining NWR oocytes, but, in the future, gametes could be obtained also from stem cell-associated techniques (5).

Value Beyond the Project

Beyond its immediate use in the project, carrying out the procedure conveys scientific, conservation, welfare, and social values. The refinement of techniques and protocols, the acquisition of new data, and the recurring veterinary screening of the animals can lead to technological and scientific improvement, which, in turn, may have positive repercussions on other conservation efforts. Moreover, the collection of biomaterials from the endangered taxa for cryopreservation has a scientific and conservation value independent from the project goals, due to its insurance value—meaning with this latter expression, the value inherent in the possibility that in the future the conserved biomaterial could be used for scientific or conservation purposes in ways unknown today or not yet developed. Given the international nature of the project, carrying out the procedure fosters knowledge transfer and the development and strengthening of links between people, groups, and institutions interested in conservation.

Risks and Costs

The main risks of the procedure are that it may harm the animals involved. This would be a problem from the point of view of each of the three value dimensions considered: the animal welfare dimension, for obvious reasons, but also the conservation dimension, since an accident could diminish the chances of saving the taxa, and the human dimension, since many people, for various reasons, care about the wellbeing and health of the two animals.

Specifically, there are three potentially critical factors in the procedure: ovarian stimulation involves a series of injections with a GnHR agonist which may accelerate pre-existing

pathologies in certain individuals; the transrectal nature of the operation, which despite all caution may lead to enhanced infection risks; general anesthesia, which, while reducing the need of mechanically restraining the animal, can give rise to complications.

In the event of a complication due to the procedure, the negative repercussions would be manifold. In addition to the harm caused to the animal involved, the possible economic damage to the owners should be considered. Other negative repercussion will be the suffering caused to people who had established relationships of some kind with the animal and the damage to the image of the project and for the entire community of conservationists.

Public View

The use of biotechnologies is particularly debated since, according to some, it distorts some fundamental aspects of the mission of conservation. Conservation ARTs could be accused in this sense to be a form of technofix, of creating a moral hazard, and of being a manifestation of hubris.

Evaluating the Conflicts and Addressing the Concerns

After building up the EM and mapping the factors involved in the assessment, the main goal of the ethical analysis is to evaluate the conflicts and to address the concerns. Conservation efforts raise inevitable conflicts, as their implementation usually affects different value dimensions and has to deal with complex sets of, often, irreconcilable demands. This is the case also with the OPU procedure that we have been analyzing, especially concerning two issues: the welfare of the involved animals, and the idea of conservation it may convey.

Concerns for the Welfare and Lives of the Animals Involved

Actions necessary for the conservation of the NWR taxon may be detrimental, in case of an accident or complication, to the welfare of the rhinoceros involved in the project, or even pose a threat to their life. However, refusing to intervene would mean failing the duty to conserve

important elements of the biodiversity of Earth. A possible radical solution to this conflict would be to rely on an alternative biotechnology, such as the production of gametes from induced pluripotent stem cells. In this way, the same results could be obtained without the risks associated with the OPU procedure. The trouble with this solution, however, is that at the moment, this technology is not yet available for rhinoceros. Due to the age of the remaining NWR, waiting could mean losing the possibility of having both females alive when the first calf will be born, further limiting the generational transmission of skills and cultural traits. While behaviorally the NWR and SWR do not seem to differ decisively from each other, there are some unique elements in the repertoires of the two subspecies. In particular, eating habits seem to differ (46), as well as, to some extent, vocalizations (101). The role played by generational transmission in the expression of these behaviors is not clear, and it is also not clear whether they could be eventually recovered and passed to the future generations of NWR. Nonetheless, it would be unwise to miss this last opportunity, especially considering that the eldest of the two females, Najin, was able to carry out a pregnancy and rear an offspring.

The only viable solution, at present, is to reach an acceptable compromise among the different value dimensions involved. This means that no value demands can be disregarded, or on the contrary, assumed as the only important one to follow. For instance, however valuable we may consider the conservation effort for the survival of NWR, it cannot overrule the basic requirements of animal welfare. At the same time, it must be accepted that as veterinarian procedures, OPU interventions necessarily involve some level of risk concerning the life and the welfare of the animals.

Ovarian stimulation is the first potentially problematic issue of the procedure and should be avoided where there are concrete risks to promote tumor growth in the reproductive tract and induce malignancy.

A second issue is anesthesia, which can give rise to dangerous side effects or even results in the death of the animal. To cope with the matter, the OPU procedure on NWR makes use of an anesthesia protocol specifically devised (102). The main advantage of this protocol is that it is

etorphine-free, preventing in this way all the possible side effects associated with this drug, which can be rather severe for the cardiovascular and respiratory systems (88–90), as well as risks of accidental exposure. The protocol is based on four different drugs (butorphanol tartrate, detomidine hydrochloride, midazolam hydrochloride, and ketamine hydrochloride), which interact synergistically with one another, enabling a reduction of their dosage and hence their possible side effects. Moreover, each of these drugs—except for ketamine hydrochloride—has an antidote, and their effects can be reversed completely.

Butorphanol-based protocols are considered a valid alternative for immobilizing white rhinos (103) and have been shown to produce less respiratory depression and hypoxia (104). Currently, this protocol has been used on more than 500 rhinoceros of different species—both in captive, wild, or semi-wild conditions—and has shown no side effects even if repeatedly used in the same individuals. Consecutive repetition of the protocol makes it possible to better tailor it to the peculiarities of the specific animal. Moreover, the unnecessary use of anesthesia—something to be avoided especially in old animals—can be minimized by proceeding with a preliminary ultrasound screening when the animal is only lightly sedated (i.e., standing sedation), and then choosing whether to continue and proceed into full recumbent anesthesia or terminate the procedure. While frequencies of the procedure similar to those in use with cattle, buffalo, and horses are ruled out, these safer anesthesia protocols allow for the repetition of multiple OPUs on the same individual within a reasonable lapse of time (4).

Finally, a third issue comes from the transrectal puncture which is required to reach the ovaries. Even if restricted to a single penetration of the rectal wall, this puncture still poses a slight risk of infection due to the potential contamination of the puncture needle (47). To mitigate this risk, the rectum of the animal is thoroughly cleaned and disinfected before the procedure, following operative standards similar to those used in human medicine prior to colon resection (47).

In order to check each application of the procedure, an ethical self-assessment through a dedicated tool, ETHAS (105), is practiced before each intervention.

Table 6 recaps all the animal welfare issues and the minimization strategies adopted.

Table 6. Welfare issues and minimization strategies

Procedure	Animal welfare issues	Minimization strategy for the con
Ovarian stimulation	Ovarian stimulation increases the number of available follicles, helping in this way to maximize the collection of oocytes per anesthesia and reducing the number of interventions as much as possible. Con: Injections can be stressful for the animals. Ovarian stimulation may accelerate the progression of certain existing genital tract pathologies.	Exclusion of animals with severe genital tract pathologies from the OPU program.
Full anesthesia	Full anesthesia removes the necessity for mechanically restraining the animals during the procedure—with all the associated risks of injury. Con: May cause severe complications such as aspiration, respiratory depression, hypoxemia, hypertension, pulmonary shunting and ventilation/perfusion mismatch.	Specifically designed ethorphine-free protocol already tested on 500+ animals. The protocols employ four different drugs in order to lessen their individual dosages. For each drug with the exception of ketamine hydrochloridre a specific antidote is available to immediately reverse the effects. Preliminary ultrasound screening may remove possibility of unnecessary use of anesthesia. Ovarian stimulation, maximizing the number of oocyte recovery for each intervention.
Transrectal ultrasound-guided oocyte recovery	Con: Non sterility of the procedure, with the risk of infection.	Cleaning and disinfection of the rectum prior the procedure adopting operative standards from human medicine. Ovarian stimulation, maximizing oocyte recovery for every intervention.

Concerns About Conservation ARTs

Conservation ARTs push us far from a model of conservation where our main goal is to limit our interaction with the natural processes. Conservation ARTs, in fact, redefine one of the most paradigmatic of the natural processes, reproduction. In this regard, conservation ARTs may be

accused to be hubristic, to be a technofix, or to create a moral hazard.

Without pretending to exhaust the complexity of these arguments, it can be nevertheless noted that they are often used to prove too much with too little. The hubris argument, for instance, is often grounded on the idea that some technologies— particularly those that, by breaking new ground, run the inevitable risk of producing unexpected consequences—may create more problems than they address, and eventually, may even lead to catastrophe. When this argument is used to urge caution, there is nothing suspicious in it, because, in applying a new technology, the risks are often real. However, if the argument is generalized to claim that every application of new technology, even when adopting the necessary measures and protocols, will produce uncontrollable negative consequences, then it is no more plausible.

Concerning the technofix argument, there could be few objections to the fact that conservation ARTs are an attempt to reverse the effects of an ongoing process, that is, human-caused extinction, through the use of technology. This remark, however, can be interpreted in two senses. In the first sense, it can be interpreted as an invitation to not lose sight of the causes that led to the current state of affairs regarding the NWR. This is important. Trying to reverse the decline of a population cannot be done without removing the original causes that led to this situation. Addressing the causes is, in this sense, a necessary condition for success. In a second sense, the previous remark can be interpreted as stating that there is something inherently wrong in working on the effects because this is not sufficient. This is misleading because something not sufficient might still be necessary. In the case of the NWR, for instance, the extinction clock cannot be brought back just by solving the issues that set it into motion, as reverting the population decline is also needed.

The moral hazard argument is based on the claim that having an easy way to revert extinction could make us even more reckless in our attitude toward biodiversity and the environment. To use an analogy, having a lifeboat at our disposal could make us more foolhardy in driving the boat. Again, if this argument is used to caution against the possible perils of new technology, it is sound. If it is used instead to convince us to abandon the technology, it is implausible.

Lifeboats may make us more risk-prone, as much as car insurance is said to make drivers less prudent. However, people just do not stop using them because they might increase the risk of incidents. This is because their benefits, in case of an incident, are higher than the costs associated with the risks they may create. The same happens with conservation ARTs: their utility far surpasses the moral hazard they might pose by granting us with a certainly not easy, but nevertheless possible, way to reverse extinction.

Conclusion

Ethical analysis provides us with a way to reflect on a procedure or on a project and it is a necessary step in making its responsible implementation possible. This study presented a frame for the ethical analysis of conservation ART procedures based on the use of the EM to collect the ethically relevant factors to identify issues and value conflicts. The advantages offered by the use of the EM are manifold. In particular, the EM makes it possible to collect and organize the elements, starting from several principles and stakeholders, allowing for a more balanced approach in evaluating complex moral scenarios where different needs, interests, and ethical concerns may conflict.

The focus of the frame presented here is on procedures, and as such, it cannot replace a structured assessment of projects. Although it includes among its requirements the analysis of the general goals and of the context of the procedure, it should not be confused either with an overall evaluation of conservation ARTs or with a general scheme for evaluating complex projects. This does not undermine its utility. The acceptability of the procedures—with respect to the mission of conservation, the welfare of the animals, the people involved, and the public opinion—is an important aspect to discriminate between those projects that are conducted responsibly and those that are not. As applications of conservation ART to endangered taxa will become more and more common, the need to explore their ethical implications becomes increasingly important.

The case study we analyzed is exemplary in this sense. Although the analysis is specifically built around the OPU procedures carried out on white rhinoceros in the context of the conservation

efforts to save the NWR, the EM can be used as a template for analyzing ART procedures performed on other rhino taxa and other endangered species. It is rather plausible that the standard scenario of ART procedures administered to rhinoceros or other species for conservation efforts will be simpler than this case. However, this would not reduce the need to carefully address the ethical issues involved.

Ethics statement

The animal study was reviewed and approved by Internal Committee for Ethics and Animal Welfare of the Leibniz-IZW—Approval No: 2019-01-02.

Author contributions

PB, TH, and BM: conceptualization. PB: methodology, original draft writing and preparation, and visualization. CG and SH: data curation. PB, BM, TH, SH, FG, RH, CG, JS, IP, MS, GL, SC, SS, JZ, SN, SM, LK, IL, PO, and DN: editing and reviewing. BM: supervision and project administration. TH: fund acquisition. All authors contributed to the article and approved the submitted version.

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106.

1.2 An ethical assessment tool (ETHAS) to evaluate the application of assisted reproductive technologies in mammals' conservation: the case of the northern white rhinoceros (*Ceratotherium simum cottoni*)

This part of my Ph.D. has been developed as member of the ethical team of the international BioRescue consortium, and the following chapter is an adaptation of:

de Mori, B., Spiriti, M. M., Pollastri, I., Normando, S., Biasetti, P., Florio, D., Andreucci, F., Colleoni, S., Galli, C., Göritz, F., Hermes, R., Holtze, S., Lazzari, G., Seet, S., Zwilling, J., Stejskal, J., Mutisya, S., Ndeereh, D., Ngulu, S., Vigne, R., Hildebrandt, T.B. (2021). An ethical assessment tool (ETHAS) to evaluate the application of assisted reproductive technologies in mammals' conservation: the case of the northern white rhinoceros (Ceratotherium simum cottoni). Animals, 11(2), 312. https://doi.org/10.3390/ani11020312

Simple Summary

Applying assisted reproductive technologies (ARTs) to the conservation of endangered species may be the only way to save them from extinction. However, ART application can raise relevant ethical issues and could benefit from a comprehensive ethical assessment. Unfortunately, there is a lack of attention to the topic in the scientific literature and, to our knowledge, there is no tool for the ethical assessment of ARTs in the context of conservation that has been described. In the present paper, we show the effects of applying a dedicated ethical self-assessment tool, the Ethical Assessment Tool (ETHAS), to ovum pick-up and *in vitro* fertilization procedures performed within the BioRescue project. The BioRescue project is an international enterprise using ARTs to save the northern white rhinoceros from extinction. The situation of the northern white rhinoceros is particularly critical as there are only two individuals of this subspecies still alive and they are both infertile females. The application of the ETHAS to the procedures contributed to the overall acceptability of the project and improved communication among the

project's partners. In turn, the tool itself was also refined through an iterative consultation process between experts (both ethicists and scientists) and stakeholders.

Abstract

Assisted reproductive technologies (ARTs) can make a difference in biodiversity conservation. Their application, however, can create risks and raise ethical issues that need addressing. Unfortunately, there is a lack of attention to the topic in the scientific literature and, to our knowledge, there is no tool for the ethical assessment of ARTs in the context of conservation that has been described. This paper reports the first applications of the Ethical Assessment Tool (ETHAS) to trans-rectal ovum pick-up (OPU) and *in vitro* fertilization (IVF) procedures used in a northern white rhinoceros (*Ceratotherium simum cottoni*) conservation project. The ETHAS consists of two checklists, the Ethical Evaluation Sheet and the Ethical Risk Assessment, and is specifically customized for each ART procedure. It provides an integrated, multilevel and standardized self-assessment of the procedure under scrutiny, generating an ethical acceptability ranking (totally, partially, not acceptable) and a risk rank (low, medium, high), and, hence, allows for implementing measures to address or manage issues beforehand. The application of the ETHAS to the procedures performed on the northern white rhinoceros was effective in ensuring a high standard of procedures, contributing to the acceptability and improved communication among the project's partners. In turn, the tool itself was also refined through an iterative consultation process between experts and stakeholders.

Introduction

In the present global scenario, where an accelerated rate of extinction is paired with a severe decline in populations' abundance in surviving species [1,2], assisted reproductive technologies (ARTs) can make a difference in biodiversity conservation. ARTs can raise the chance of success of conservation breeding programs by both overcoming infertility issues and optimizing genetic management, avoiding inbreeding (or outbreeding) depression and risks of transmission of inherited diseases [3–5].

ARTs, in fact, may offer the only chance for survival of many endangered species with very fragmented populations or only few extant individuals. In this case, ARTs can be employed not only to boost the number of offspring, but also to enhance the genetic exchange between the fragmented populations (living both in situ and ex situ) without the need of actually translocating the animals [6], ARTs can also enhance the genetic exchange between living and dead generations by using gametes stored in cryobanks [7] or, in what could be a possible near-future development of this biotechnology, produced from stem cells [8].

While ARTs are a robust opportunity in the conservationist's toolbox—and one which promises to become increasingly important in the future—their application may raise several ethical issues. The use of ARTs can raise ethical concerns also in human medicine, some of which can be still valid when ARTs are applied to non-human animals, but many of the issues raised by the application of these technologies in conservation breeding projects are more specific [9–11]. These may range from issues also common in applications of ARTs to livestock [12–14] to more specific issues tied to the particular context of biodiversity conservation. For instance, ARTs need species-specific optimization in order to be successfully employed, and this, in turn, depends on detailed knowledge of the reproductive biology of the species involved [4]. Such knowledge may be difficult to obtain in already endangered species, due to the limited numbers of available individuals for research and the potential difficulties in accessing them [15]. In the end, its pursuit may pose several dilemmas to scientists and conservationists intentioned to both safeguard the remaining individuals of a species and obtain enough information for a last attempt to reverse its decline. It could also be claimed that important resources—in terms of time, space, people, competencies, and funding [16], which are needed to implement conservation projects involving ARTs, from the first step of species-specific optimization of the techniques to the breeding and reintroduction steps—could be perhaps better allocated to other more traditional forms of biodiversity conservation. Moreover, from a more theoretical perspective, applying ARTs could be seen as an exemplary case of "technofix" [11,17], that is, the short-sighted use of technology as a way to sort out the outcome of morally problematic activities instead of

addressing their causes, or as an apparently "easy" solution to the decline in wildlife populations, with the risk of inducing complacency in the problem.

Above all, a crucial source of ethical concern regarding ARTs in biodiversity conservation is animal welfare. Many applications of ARTs require manipulation of live animals and, in some case, invasive procedures, with real risks for their welfare. This is of course also true for farm animals, where the issue has not received enough attention (for instance, [18,19]), but is further exacerbated in wildlife, where at least three factors intervene to complicate the matter. The first is the experimental characters of many ARTs applications to wildlife, with procedures less established than in livestock and which often stand in a gray area between research and veterinary practice. The second is our knowledge on animal welfare science, which, again, is scarcer in wildlife than in farm or laboratory animals. The third concerns manipulation of the animals. While livestock and, in general, domestic animals are more accustomed to being manipulated by people, operating on wildlife may be more stressful for the animals involved (and also for the staff performing the procedures) and may be more demanding in terms of restraint, sedation, or anesthesia. Moreover, this higher toll exacted in terms of animal welfare may be more difficult to mitigate, since excessive conditioning of the animals involved in the procedures could be undesirable due to the need for minimizing the effects of captivity [20].

In general, when an ethical assessment of a procedure involving individual animals has to be carried out, the golden standard would be a systematic project evaluation, requiring, among other things: (i) a risk assessment; (ii) an assessment of welfare conditions and pain, suffering, distress, and lasting harm imposed on the animals; (iii) a harm–benefit evaluation; and (iv) the application of the 3Rs (Replacement, Reduction and Refinement) [21]. This standard is, at least in theory, systematically applied when research projects involving laboratory animals are submitted to ethical committees for evaluation. With regard to wildlife, however, this standard evaluation is not performed systematically. Yet this evaluation is crucial, especially for projects involving ARTs.

Risk assessment, for instance, should be considered essential in these cases. Application of ARTs to wildlife and their biomaterial entails accepting a certain grade of uncertainty. This requires a prior definition of the ethically tolerable risk threshold for the procedures, which can be conducted only by performing a detailed risk analysis, based on traditional risk analysis [22], specific animal welfare [23] and ethical risk analysis [24], and application of the precautionary principle [25–28].

The assessment of potential pain, suffering, distress, and harm, alongside general welfare conditions of the individual animals involved in the procedure, should also be considered essential. However, pain, suffering, distress, harm, and, in general, the welfare of the individual animals have traditionally played a secondary role in biodiversity conservation. This is partly due to the fact that the goals of biodiversity conservation and of animal welfare are conceptually distinct and may sometimes diverge, since the former is mainly focused on species, whereas the latter is focused on individuals [29–31]. Nevertheless, excessive divergence may remove societal support for conservation projects [32,33]. Moreover, animal welfare is a crucial factor in the success of conservation breeding and reintroduction programs [20,34]. Yet, as already noted, the assessment of wildlife welfare may be harder to obtain. Knowledge on the issue is lacking if compared to laboratory animals. This is both due to fewer research works on the former subject than on the latter and to the larger number and diversity of wild vertebrate species compared to the few taxa employed in laboratory research [35]. These difficulties, however, do not remove the need to carefully assess the general welfare conditions and the specific potential pain, suffering, distress and lasting harm imposed on the animals during the application of the ART procedures.

The third important requirement is harm–benefit analysis. Again, while this is nowadays routine in the ethical assessment of laboratory projects involving animals [36], it is instead underrepresented in wildlife studies. In particular, harm–benefit analysis has been rarely applied to evaluate the impact on the health and welfare of wild animals involved in veterinary procedures aimed at safeguarding their species [37]. Nonetheless, it is progressively used to

identify costs and benefits arising from conservation projects in relation to not only their economic impact [38], but also to their positive or negative consequences for the ecosystem and the local wildlife population.

The same can be said also for the fourth requirement, the application of the 3Rs, which has been widely satisfied in laboratory research but rarely in wildlife studies, where research conditions are more heterogeneous and it is harder to standardize a methodology for its implementation as has been done in laboratory research. However, as progressively stated [39,40], the 3Rs principle is crucial also for wildlife research. For instance, replacement can be obtained with non-invasive research techniques, reduction with optimized experimental design and refinement with better methods of capture, anesthesia and handling [39].

It may be countered that conservation interventions do not qualify—at least in a full sense—as research and, as such, should not be subjected to the same stringent standards involved in laboratory research. However, as already noted, the boundaries between research and veterinary practice are often blurred when applying ARTs to conservation breeding programs. Moreover, most applications of ARTs to wildlife may take place both in research and non-research scenarios. This raises a boundary problem, as the same activity may be subjected to different ethical standards of evaluation when performed in different contexts. To solve this inconsistency, it has been suggested that far from relaxing our ethical standards on research, we should instead extend them to all similar activities [41,42].

For all these reasons, conservation projects incorporating ARTs should be carefully scrutinized in order to evaluate their ethical acceptability, using the highest procedural standards and compliance with best practices and regulations as landmarks. Currently, despite the increasing interest in the use of ARTs in conservation, there is little attention to ethical assessment and, to our knowledge, there are no tools to evaluate the specific risks and ethical aspects involved. A simple search on Scopus (https://www.scopus.com/), with “ethical assessment” AND “reproduction” and “wild” and “animal” as keywords run in December 2020, gave no results. One of the reasons for this result could be that, with ARTs being applied to conservation breeding

projects often in the gray area between clinical practice and research, their use in such context often does not require external ethical approval. It is therefore even more important that the practitioners and the researchers involved in these types of projects are able to evaluate the potential ethical relevant issues spanning from the procedures they use themselves. One way to enable practitioners and researchers to evaluate their procedures is to provide them with a comprehensive and customizable tool for the self-assessment of such procedures, which, once developed by experts with an ethics background (specifically, in applied ethics related to conservation and animal welfare), can be used also by people lacking such background. Self-assessment could also be an important step in preparation for an external overall evaluation of the ethical acceptability of a project and could help scientists to be proactive and to scrutinize the ethical issues surrounding their work [43].

In this paper, we present the self-ethical assessment of two ART procedures performed in the context of a conservation breeding program aimed at avoiding the extinction of the northern white rhinoceros (*Ceratotherium simum cottoni*—NWR). The procedures involved both southern white rhinoceros (SWR) females in European zoos and the last two surviving NWRs. The assessment was preformed using a self-assessment tool explicitly designed for conservation breeding programs, the Ethical Assessment Tool (ETHAS), as customized for the self-assessment of ovum pick-up (OPU) and *in vitro* fertilization (IVF) procedures. The aim of the study was to investigate both whether applying the tool could contribute to ensuring a high standard and improvement of procedures being assessed and, at the same time, how applying the first version of the tool in actual field conditions contributes to shape and improve the tool itself.

Materials and Methods

The Case

The NWR, a subspecies of the white rhinoceros (*Ceratotherium simum*), once ranged over much of the savannah of Central Africa [44–46]. However, between the 1970s and the 1980s, the wild population was reduced to only 15 individuals, and there have been no reported signs of their

presence in the wild since 2007. Nowadays, it is declared as “possibly extinct in the wild” [47], as the only remaining individuals live in captivity. The last remaining individuals are two females, Najin and Fatu, who are under constant surveillance at Ol Pejeta Conservancy, in Kenya, and cannot have a viable pregnancy due to health and age-related issues. Najin is 31 years old and has a large ovarian tumor on her left adnexus. Moreover, she has very weak hind legs due to bilateral alterations of the Achilles tendons. Her 20-year-old daughter Fatu has developed untreatable degenerative endometriosis of unknown cause over her entire uterus [48]. Therefore, the only chance to save this iconic subspecies from the brink of extinction is to utilize ART procedures, using *in vitro* embryos gestated by recipient mothers of the sister subspecies—the southern white rhinoceros (*Ceratotherium simum simum*—SWR). In order to produce embryos, however, gametes have to be obtained first. During the last two decades, scientists have collected the semen from four NWR bulls and cryopreserved it in three different cryobanks [48]. No oocytes, instead, have ever been stored because of their low permeability to cryoprotectants and consequent susceptibility to chilling [49]. This means that ovum pick-up (OPU) has to be repeatedly performed on the two surviving females, in order to obtain viable oocytes, which are then sent to a specialized laboratory for incubating, maturing and performing *in vitro* fertilization (IVF), in order to obtain viable embryos. The embryos are then stored in liquid nitrogen, until transferred into an SWR recipient mother. OPU on Najin and Fatu was performed for the first time on August 22th, 2019, in collaboration with the Kenya Wildlife Service (KWS), and has been repeated three more times. Despite the difficulties caused by the COVID-19 pandemic, at present, there are five embryos ready for transfer.

When conducting an ethical assessment on ART procedures involving Najin and Fatu, the health-related issues of the two individuals are likely to be very relevant both because, as already said, they prevent the two animals from having a viable pregnancy and they impact on their welfare, mainly by modifying the risks that ART procedures create for the involved animals. In rhinoceroses, in general, OPU needs full anesthesia [50,51], with the animal lying down, and thus it may be a risky procedure even in healthy animals [48,52,53]. The scientific literature and

best practices show that rhinoceroses quickly recover from ovum pick-up [54,55]—as fast as farm animals—making repeated anesthesia possible even within a short time period [51,56,57]. The health situation of the two NWRs may alter the risks posed by repeated anesthesia because their chronically ill status might affect their resilience to the procedure. However, the fact that they suffer from health issues increases the importance of being able to perform OPU with a higher frequency on them, in order to have more chances to succeed in saving the species from utter extinction, since their health issues might adversely affect their life expectancy and thus the time available for scheduling OPU.

Given the complexity of the ethically relevant issues involved, a sub-project dedicated to the development of a specific ethical self-assessment tool which could be used in mammalian conservation breeding programs was created within the BioRescue project—the international consortium led by the Leibniz Institute for Zoo and Wildlife Research of Berlin (Leibniz-IZW) and comprising the Czech Dvur Králové Zoo, Avantea laboratory, Max Delbrück Center for Molecular Medicine (MDC), Kyushu University and Padua University (and having the support of other international partners), which is in charge of the whole project that involves Najin and Fatu and aims at avoiding the final extinction of the northern white rhinoceros.

The Tool (ETHAS)

The Ethical Assessment Tool (ETHAS) is a flexible and customizable instrument for the ethical self-evaluation of specific ART procedures applied to mammals in biodiversity conservation projects. It includes and integrates with each other risk assessment (general, ethical and welfare), pain/distress/welfare evaluation, harm–benefit analysis and the 3Rs tenet application. As already stated, self-assessment tools help scientists to be proactive and to scrutinize the ethical issues surrounding their work and are preliminary for an external overall evaluation of the ethical acceptability of a project [43]. Their implementation fosters dialogue between all participants and may lead to the actual improvement of the procedures. Moreover, routinely performed ethical self-assessment helps scientists to comply with ethical principles, best practices with

animals, relevant legislation and authorizations and ethical approval [35]. Self-assessment cannot replace ethical assessment by an external committee, but it contributes both to the final acceptance of the project, by anticipating its possible ethically critical issues (and hence allowing for timely and comprehensive design of mitigation strategies), and to the communication of its results to the general public.

ETHAS is based on checklists, a tool commonly used in medicine and other fields to identify errors, ameliorate operational standards and comply with best practices [58,59]. Checklists are a valuable tool for self-assessment. Their use improves research results and makes them easier to be communicated, contributing to the responsible conduct of research, thereby increasing its public acceptance [35,43]. Moreover, they can be used by both experienced and inexperienced personnel alike, and they are easily understandable and verifiable [59].

ETHAS's checklists aim to combine risk assessment with ethical acceptability assessment. Risk assessment is a crucial phase of risk analysis, and therefore it is very important for the overall ethical acceptability of wildlife conservation projects. As it is known, risk analysis is a three-step process: (i) risk evaluation/assessment, (ii) risk management and (iii) risk communication [60,61]. It allows a standardized, repeatable, transparent and documented evaluation of the risks posed by a course of action or a chain of decisions [62]. The use of ARTs on wild animals entails the acceptance of a certain level of risk, but this level must conform to the "as low as reasonably applicable principle" (ALARP) [63].

Therefore, the general frame of the ETHAS tool is based on two integrated checklists for self-assessment, the Ethical Evaluation Sheet (EES) and the Ethical Risk Assessment (ERA). Each ERA item is conceptually linked to a corresponding part of the EES checklist, which comprises, among others, all the relevant ethical aspects that are investigated in ERA. The link is reported in a column with an alphanumeric code.

There are customized EES and ERA versions for each ART procedure, but all share some common features. These constituent checklists of both EES and ERA have been developed on the basis of the current literature and best practices guidelines and refined through an iterative

consultation process between experts (both ethicists and scientists) and stakeholders, which is still ongoing in the present stage of the project. They merge risk analysis, based on a combination of traditional, animal welfare and specific ethical risk assessments, with ethical analysis, based on pain/distress/welfare evaluation, harm– benefit analysis and the 3Rs tenet application, with the aim of defining the overall ethical acceptability of the procedure under assessment.

Ethical Evaluation Sheet (EES)

The Ethical Evaluation Sheet (EES) highlights potential ethical issues arising from the ART application. As with corresponding tools for the ethical assessment of research projects with laboratory animals [43,64–66], the general frame of EES consists of four main sections of investigation: (a) Documents; (b) Harm–benefit evaluation; (c) Procedure quality evaluation; and (d) Scientific team quality evaluation. For each specific ART procedure, it is necessary to detail a certain number of items within these main sections. In the first trial, the EES for the OPU procedure consisted of a total number of 83 items, whereas the IVF-lab EES consisted of 64 items. However, since some items are made up of sub-items, the total possible answers counted in the final score can be more. Regarding the OPU EES, the total number was 88, while in the IVF-lab EES, it was 81. After the revision of some items, detailed in Section 3.2, a second version of both the OPU and the IVF-lab EES was developed. The second version of the EES for the OPU procedure consisted of a total number of 86 items, with a total number of 91 items and sub-items, whereas the second version of the IVF-lab EES had 66 items, with a total number of 83 items and sub-items.

Table 1 shows the general structure of the EES checklists for OPU and IVF procedures in more detail and reports the scientific sources of information used in their development.

Table 1. Ethical Evaluation Sheet sections and bibliography.

EES Sections and Sub-Sections	Number of Items (Sub-items) OPU EES		Number of Items (Sub-items) IVF-Lab EES		Bibliography
	1st Trial	2nd Trial	1st Trial	2nd Trial	
A) Documents	11 (13)	11 (13)	9 (10)	9 (10)	[21,39,43,67–73]
B) Harm–benefit evaluation of the procedure					
B1) Benefit evaluation	12 (14)	12 (14)	7 (7)	7 (7)	[36,64–66,69,74–81]
B2) Harm evaluation	8 (9)	8 (9)	4 (8)	4 (8)	
C) Procedure Quality Evaluation					
C1) Pre-screening consideration	6 (6)	6 (6)	6 (6)	6 (6)	[21,36,39,40,54,64–66,75–82]
C2) Procedural steps evaluation	3 (3)	3 (3)	5 (5)	5 (5)	
C3) 3Rs evaluation (replacement, reduction, refinement)	23 (23)	23 (23)	14 (21)	14 (21)	
D) Scientific team quality evaluation					
D1) Team and teamwork	13 (13)	14 (14)	12 (17)	12 (17)	[62,64,76]
D2) Equipment	5 (5)	7 (7)	4 (4)	6 (6)	
D3) Laboratories and biobanks	2 (2)	2 (2)	3 (3)	3 (3)	
E) Final ethical evaluation of the procedure	11 (11)	11 (11)	9 (9)	9 (9)	[76]

The EES is designed to be filled in only once (unless the procedure's protocol is changed) before to start the procedures. In the case of the procedures performed during the present study, as it was a phase in the development of the final version of the tool, the EES was filled in by a member of the BioRescue team with an ethical background in applied ethics in conservation and animal welfare. However, as underlined in the Introduction, in the final version of the tool, any member of the team performing the procedures will be able to fill in the EES, without the need of a specific ethical background. During the EES compilation, it is asked to answer "yes" or "no" to all items, depending on whether the requirements are met or not. Moreover, for some EES items, it is required to add further information to explain the answer. The EES is evaluated using a semi-quantitative scoring model in which the answers "yes" or "no" assume the value of 0 and

1, respectively. The sum of the items' outcome divided into three homogeneous ranges defines the rank of the ethical acceptability of the procedure: not acceptable, partially acceptable, acceptable. Therefore, the final score obtained from the EES compilation identifies one of the three acceptability ranks. Table 2 describes the EES final score for the OPU and IVF procedures performed in the present study.

Table 2. Acceptability ranking and scoring of the ovum pick-up (OPU) and *in vitro* fertilization (IVF-lab) Ethical Evaluation Sheets (EESs) applied in the present study.

Acceptability Ranking	Score in OPU EES		Score in IVF-Lab EES	
	1st Version	2nd Version	1st Version	2nd Version
Totally acceptable	0–29	0–30	0–27	0–27
Partially acceptable	30–58	31–60	28–54	28–55
Not acceptable	59–88	61–91	55–81	56–83

The identified acceptability level that represents the outcome of the EES assessment (defined as the first review level) defines the degree of the procedure acceptability. In case of a partial or not acceptable result in the ethical assessment, detected with the first review level, each section of the EES checklist is assessed individually. This second review level identifies at which section of the procedure corrective actions need to be planned. Finally, a third review level allows identifying the items whose requirement is not met and, therefore, the critical issues of the procedure to be reviewed before the procedure begins.

Ethical Risk Assessment (ERA)

The ERA checklist is specifically customized for each procedure under scrutiny by identifying the appropriate phases for risk assessment. The scientific literature on ARTs has been revised to analyze, in detail, each step of the OPU and IVF procedures and detect possible hazards and ethical risks whose occurrence could negatively impact on the animal welfare, staff safety and procedure outcome [83]. As shown in Table 3, the OPU ERA is composed of five different phases: A) Identification of the individual/s, welfare assessment and procedure planning; B) Ovarian stimulation protocol; C) Anesthetic procedure for oocyte recovery; D) Oocyte recovery by transrectal procedure; and E) Gametes packaging. The total number of items in the OPU ERA first version was 52, while in the second, it was 56. Since some items are made up of sub-items, the total number of the first version was 91, while that of the second one was 101. Table 3 shows the OPU ERA checklist in more detail and reports the scientific sources of information used in its development.

Table 3. OPU Ethical Risk Assessment (ERA) phases and bibliography.

OPU Ethical Risk Assessment Phases		Number of Items (Sub-Items) 1° Version	Number of Items (Sub-Items) 2° Version	Bibliography
A)	Identification of the individual/s, welfare assessment and procedure planning	17 (34)	19 (36)	[19,67,84–88]
B)	Ovarian stimulation protocol	6 (8)	6 (8)	[50,54,89]
C)	Anesthetic procedure for oocyte recovery	10 (18)	13 (27)	[50,52–57,89–91]
D)	Oocyte recovery by transrectal procedure	12 (20)	11 (19)	[50,54,90,92–94]
E)	Gametes packaging	7 (11)	7 (11)	[95–97]

The IVF-lab ERA, instead, as shown in Table 4, is composed of nine phases: (A) Laboratory quality assessment and specimens processing; (B) Gametes shipping to the laboratory; (C) Gametes biobanking; (D) Gametes preparation for ICSI; (E) Intracytoplasmic sperm injection (ICSI); (F) Embryos culture; (G) Embryos cryopreservation and biobanking; (H) Embryos packaging; and (I) Embryos shipping. The total number of items in the IVF-lab ERA was 72. Since some items are made up of sub-items, the total number was 103.

Table 4. IVF-lab ERA phases and bibliography.

IVF-Lab ERA Phases	Number of Items (Sub-Items)	Bibliography
A) Laboratory quality assessment and specimens processing	17 (32)	[98–101]
B) Gametes shipping to laboratory	7 (8)	[7,54,102–104]
C) Gametes biobanking	7 (8)	[7,102,105,106]
D) Gametes preparation for ICSI	13 (16)	[54–107]
E) Intracytoplasmic sperm injection (ICSI)	6 (6)	[54,92,107,108]
F) Embryos culture	7 (7)	[54,109]
G) Embryos cryopreservation and biobanking	4 (11)	[54,102,110]
H) Embryos packaging	4 (7)	[109]
I) Embryos shipping	7 (8)	[109,111]

Each item and sub-item of the ERA checklists analyzes an element of the procedural step which could cause a hazard to the success of the phase under assessment. For each item, it is required to record a “yes” or “no” whether the requirement of the item is satisfied or not. Depending on the characteristics of the requirement and on the severity of the consequences associated with the hazard scenario, each item is scored differently (Table 5). For example, the consequences associated with a failure highlighted with items in phases A, B, C and D of the OPU ERA have different effects. Non-compliance with operational or animal management requirements has a more significant impact on animal welfare than non-compliance with operational instructions or documentary, structural, instrumental and environmental requirements (Table 5). The items of

phase E of the OPU ERA have been evaluated with the risk categories of the IVF-lab ERA due to the consequences of the hazard impact on the gametes' safety. In the IVF-lab ERA, three scoring ranges were defined on the basis of the type and severity of the possible outcomes that the hazard scenarios could have on gametes and embryos.

Table 5. Description of risk categories and corresponding score used for phases A, B, C and D the OPU ERA and for phase E of the OPU ERA and all phases (A–I) of the IVF-lab ERA.

Phases	Categories	Characteristics of the Requirement	Score
OPU ERA (phases A–D)	Low	Documents, procedures, operating instructions, etc.	1
	Medium	Structural, instrumental and environmental requirements.	2
	High	Operational requirements.	3
OPU ERA (phase E) and IVF-lab ERA	Low	Factors affecting the process (documental and procedural support aspects).	1
	Medium	Factors related to the traceability and distribution of specimens, laboratory operator's safety, quality and availability of laboratory facilities.	2
	High	Factors related to the viability of gametes and embryos and to the instrumental requirements and the chemical reagents used.	3

The assessment uses a semi-quantitative scoring model where the risk is determined by a single value R that combines the probabilities (p) and consequences (x) associated with the occurrence of a hazard scenario [111]. The hazard scenario is identified with each ERA item. The probabilities are determined by the satisfaction or not of the item. The consequences depend on the characteristics of the requirement of the item and are classified into different levels of severity, in accordance with Table 5.

$$R = \sum_{i=1}^{n} p_i x_i$$

In the specific model, n corresponds to the number of scenarios chosen to describe the risk (number of items of the ERA checklist), pi can assume values of 0 or 1 depending on whether the requirement is met (yes) or not (no/no answer) and xi is from 1 to 3, as described in Table 5. ERA checklists are designed to be filled in each time a procedure is performed. They have to be filled in by one to three different people, depending on the procedure under assessment, with two main aims: to have an overview of the procedure and to verify, in case of more persons involved in the assessment, if communication regarding ethically relevant issues among the participants is effective. Regarding the OPU procedure, for instance, if it is executed only by the veterinary staff of the zoo or facility hosting the animals, the ERA can be filled in just by the chief veterinarian. If the OPU procedure is executed by an external veterinary team, the ERA has to be filled in both by the external and internal veterinarians and the zoo or facility managing director. In the applications of the ETHAS described in the present paper, three different participants responded to the OPU ERA for both the procedures performed: the veterinarian responsible for the BioRescue project, the local veterinarian and the managing director of the facility where the procedure took place.

Regarding the second aim—to verify if communication is effective—the three answers for each item are entered in an Excel spreadsheet, and the modal value that allows highlighting the most frequent responses per set of answers is calculated. The sum of the modal values is divided into three ranges, identifying the three categories of risk severity (low, medium, high). On the contrary, the modal value is not necessary at all for the IVF-lab ERA because it is compiled by only one person—the person responsible for the IVF laboratory. In this case, the sum of the values of each answer is divided into three ranges, corresponding to the three risk categories (Table 6).

Table 6. Risk ranks of the OPU and IVF-lab ERAs.

Risk Rank	Score in OPU		Score in IVF-Lab
	1st Version (October 2019)	2nd Version (December 2019)	Final Version (October 2019)
Low	0–63	0–73	0–61
Medium	64–126	74–146	62–123
High	127–190	147–220	124–184

Similarly to the EES, also for the ERA, three review levels can be applied: at an overall level (risk rank, first review level), at the phase level (second review level) and at the items level (third review level). The review levels allow revising the specific application of the procedure in case of the detection of a medium or high risk rank and applying risk management and risk communication strategies.

Final Overall Evaluation (EES + ERA)

The ETHAS generates a risk rank (low, medium, high) through the ERA and an ethical acceptability rank (totally, partially, not acceptable) with the EES. The overall final evaluation (ERA + EES) is calculated by combining the acceptability ranking obtained from the EES and the risk rank obtained the from ERA (Table 7). Therefore, ETHAS overall evaluation falls into three categories:

(1) Acceptable, when the ESS results in totally acceptable and the ERA detects low risks. The assessed procedure may be accepted without further actions.

(2) Acceptable with mitigation, when the EES results in partially acceptable and the ERA detects medium risks. The assessed procedure may be accepted only if critical issues are identified and addressed and the specific application of the procedure is revised.

(3) Not acceptable, when the EES detects a not acceptable result and the ERA detects high risks. The assessed procedure may be unacceptable until further improvements are enforced to eliminate the associated ethical concerns and procedural risks.

Table 7. Ethical Assessment Tool (ETHAS) overall final evaluation, obtained by combining results from the ESS and ERA checklists.

ERA / ESS	Low Risk	Medium Risk	High Risk
Totally acceptable	Acceptable	Acceptable with mitigation	Not acceptable
Partially acceptable	Acceptable with mitigation	Acceptable with mitigation	Not acceptable
Not acceptable	Not acceptable	Not acceptable	Not acceptable

Scoring of both checklists and the overall final evaluation have to be performed by the person completing the EES.

After the risk assessment, the ETHAS enables risk management of the possible highlighted hazards. Risk management (the second phase of a risk analysis process) allows raising awareness of the potential hazards and risks and enables the sharing and acceptance of the measures to be adopted to reduce the risks. Risk mitigation actions have to be chosen taking into account: (1) the characteristics of the requirements (in terms of scoring); and (2) what is reasonable and technically possible. Moreover, risk management allows an exchange of information and opinions between the staff involved in the ART procedures. Finally, the ETHAS enables also risk communication: through an iterative process among the staff directly involved in the procedures, information and opinions on hazards and their associated risks are exchanged, allowing a transparent and overarching discussion of results.

Application of the Tool

In a preliminary phase of ETHAS development, after consulting the relevant scientific literature and best practice guidelines on OPU and IVF procedures, a draft of the checklists was designed using a bottom-up approach, by witnessing several procedures and discussing with the teams performing them the main areas identified by the scientific literature and best practices. Relevant areas, not previously found in the literature search, but found to be relevant in the practical application of the ART procedures, were also added and discussed. The OPU procedures witnessed in the preliminary phase included both procedures performed on infertile SWRs in European zoos—who were involved in the BioRescue project both for approaching their infertility problems and for protocol optimization—and those (August 2019) performed on Najin and Fatu, in order to ensure suitable consideration of the relevant specific features of these individuals (e.g., their health status, as discussed in 1.1.) in the tool. The IVF procedures witnessed were all performed at the Avantea laboratory, which up to now is the only one that produced a viable rhinoceros embryo.

The preliminary phase led to the first version (beta1) of the ETHAS customization for OPU procedures (OPU EES + OPU ERA). The complete beta1 version can be found as Supplementary Material (File S1 and S2). The beta1 ETHAS version was then applied in October 2019 during an OPU procedure performed by the BioRescue team on three sub-fertile or infertile SWR females housed in a European zoo, in order to evaluate both the effects of conducting ethical self-assessment on the application of ART procedures and to improve the beta version of the tool itself.

The application of the beta1 version led to the revisions of some items, detailed in Section 3.2, resulting in the creation of an updated version (beta2) of the OPU EES and ERA. The beta2 version was applied in December 2019, during an OPU procedure performed by the BioRescue team on the last two NWRs in Kenya. Both procedures (October and December) were performed following the BioRescue team's standardized protocols. Similarly, the first version (beta1) of the ETHAS customization for IVF procedures (IVF-lab EES + IVF-lab ERA) was first applied in

August 2019 (Supplementary Material File S3 and S4), and the second one (beta2, after the changes detailed in Section 3.2) was applied in October 2019, at the Avantea laboratory.

Results

How Applying the Tool Contributed to the Refinement of The Procedures

EES

In both the first and second assessment trials, the ethical assessment of OPU and IVF-lab resulted in "Totally acceptable" in both EESs (Table 8). However, despite this result, the EESs were investigated at the second and third review levels to examine whether there were unmet requirements and, if so, in which sections and items they were found.

Table 8. EES results. Please note that the changes detailed in Section 3.2 were already included in the EES version used for the second OPU and IVF trials.

	EES	OPU EES 1st Trial		OPU EES 2nd Trial		IVF-Lab EES 1st Trial		IVF-Lab EES 2nd Trial	
		Positive Answers	**Negative Answers**	**Positive Answers**	**Negative Answers**	**Positive Answers**	**Negative Answers**	**Positive Answers**	**Negative Answers**
A)	Documents	13 over 13	0 over 13	13 over 13	0 over 13	10 over 10	0 over 10	10 over 10	0 over 10
B)	Harm–benefit evaluation of the procedure	20 over 23	3 over 23	20 over 23	3 over 23	14 over 15	1 over 15	14 over 15	1 over 15
C)	Procedure quality Evaluation	32 over 32	0 over 32	32 over 32	0 over 32	32 over 32	0 over 32	32 over 32	0 over 32
D)	Scientific team quality evaluation	20 over 20	0 over 20	23 over 23	0 over 23	24 over 24	0 over 24	26 over 26	0 over 26
	Total	85 over 88	3 over 88	88 over 91	3 over 91	80 over 81	1 over 81	82 over 83	1 over 83

The OPU EES in the first trial received a final score of 3 over 88, while in the second trial, it received a final score of 3 over 91. In both trials, the three negative answers were detected in the "Harm–benefit evaluation of the procedure" section. The first of the three unmet requirements was related to the fact that infertility is not widespread in the SWR wild population. For this reason, even if it is fundamental to optimize the procedure for this subspecies in zoos and facilities alike, there is no wilder population that can receive a direct benefit from this process. Nevertheless, the acquired knowledge on the rhinoceroses' reproduction might turn out to be useful in the future, also for the other rhino species. The second concerns the possibility that the OPU procedure may have adverse side effects on the animal under it in case of a harmful event. Even if all the precautions are taken, the risk probability is never zero. Finally, the third one was related to the fact that any adverse event on the last two NWR females impacts this subspecies. Regarding the IVF-lab EES first trial, the final score was 1 over 81, while the IVF-lab EES second trial obtained a final score of 1 over 83. Similarly to the OPU EES, the section that contained the not satisfied requirement in both trials was the "Harm–benefit evaluation of the procedure". The specific item was related to possible adverse side effects that can lead to biomaterial damage, even if all precautionary measures were taken.

ERA

The application of the OPU ERA first version, in a European zoo in October 2019, resulted in "low risk". Checklists filled in by the three respondents were analyzed for assessing both the procedure itself and the effectiveness of communication among the participants. In particular, the assessment of the procedure itself did not find any relevant nonconformity in the procedures. All potential issues were taken into account and suitable measures were enforced to minimize risks. The only negative score was concerning "previous experience of the local team" in OPU

on rhinos, which was not a problem in itself because of the presence of the BioRescue veterinary staff, who coordinated and carried out the procedures.

When the answers of all three respondents were analyzed to assess communication, the obtained risk score was 57, over a total of 190. The “low risk” ranking notwithstanding, the second and third review levels were applied, and the ERA outcome was further investigated. Twenty items—distributed among the A and D phases—were identified. The characteristics of the requirements not met were related to “Documents, procedures, operating instructions” for 10 items and “Operational requirements” for the other 10 items. Apart from “experience of the local team”, in all these cases, the problem was that the two local respondents did not answer to some items, although the BioRescue veterinarian had, so the modal value was 0. The same was true of the whole of phase E. Thanks to the third review level, it was possible to detect that the items that recorded “no” or “no answer” were mainly related to sub-optimal explicit communication of some issues between the three main people responsible for the procedure.

The highlighted communication issues in the first version were not detected in the second one. Consequently, the OPU ERA applied in December 2019 in Kenya resulted in “low risk” with a risk score of 0 over 220. Therefore, it was not necessary to proceed with the second and third review levels (Table 9).

Table 9. Results of the first and second assessment trials using the OPU ERA checklists. Please note that the changes detailed in Section 3.2. were already included in the ERA version used for the second OPU trial and that the results shown for OPU refer to the analysis of the answers of all three respondents.

OPU ERA Phases		1st Trial (October 2019)		2nd Trial (December 2019)	
		Positive Answers	**Negative Score**	**Positive Answers**	**Negative Score**
A)	Animal selection, procedure planning and welfare	27 over 34	10 over 75	36 over 36	0 over 79
B)	Ovarian stimulation protocol	8 over 8	0 over 21	8 over 8	0 over 21
C)	Anesthetic procedure	15 over 18	7 over 37	27 over 27	0 over 66
D)	Oocyte recovery by transrectal procedure	9 over 20	23 over 40	19 over 19	0 over 37
E)	Gametes packaging	0 over 11	17 over 17	11 over 11	0 over 17
	Total	59 over 91	57 over 190	101 over 101	0 over 220

The application of the IVF-lab ERA, in October 2019, resulted in “low risk”, with a risk score of 0 over 184 (Table 10). All the requirements’ characteristics related to “Factors affecting the process (documental and procedural support aspects), “Factors related to the traceability and distribution of specimens, laboratory operator’s safety, quality and availability of laboratory facilities” and “Factors related to the viability of gametes and embryos and to the instrumental requirements and the chemical reagents used” were met for the rhinoceroses’ biomaterial safety. It was not necessary to proceed with the second and third review levels. Therefore, there was no need to perform a second assessment trial after addressing problematic issues.

Table 10. Description of IVF-lab ERA standard checklist application and results.

	IVF-Lab ERA Phases	October 2019	
		Positive Answers	Negative Score
A)	Laboratory quality assessment and specimens processing	32 over 32	0 over 54
B)	Gametes shipping to laboratory	8 over 8	0 over 14
C)	Gametes biobanking	8 over 8	0 over 17
D)	Gametes preparation for ICSI	16 over 16	0 over 28
E)	ICSI	6 over 6	0 over 16
F)	Embryos culture	7 over 7	0 over 11
G)	Embryos cryopreservation and Biobanking	11 over 11	0 over 19
H)	Embryos packaging	7 over 7	0 over 11
I)	Embryos shipping	8 over 8	0 over 14
	Total	103 over 103	0 over 184

Of course, also having established the inclusion of an ethical self-assessment in ART procedures as a routine protocol is to be considered in itself as an improvement of the procedures, as it ensures the high standards of the procedures themselves.

How Applying the Tool in Actual Field Conditions Improved the Tool Itself

As already explained, the tool is designed to be able to incorporate changes allowing it to be refined by means of consultation between ethicists, scientists and stakeholders following each application of it. After the application of the first version of the tool to the OPU procedure, some areas needing further addressing in the ERA and EES checklists were highlighted. The items added as a consequence of the process in the OPU EES and OPU ERA are shown in Table 11. The items added to the OPU EES were also added to the IVF-lab EES as they were also relevant to the IVF procedure

2910 **Table 11.** Items added to the first OPU EES, OPU ERA and IVF-lab EES standard versions to
2911 obtain the second ones.

New Added Items to OPU EES
Have the aspects related to the environmental impact of the staff travels been considered and have measures been taken to decrease it? (i.e., use train instead of airplane whenever possible, contributing to a certified carbon offset program for flights)
Have the aspects related to the environmental impact of the equipment and materials been considered and have measures to decrease it been taken?
Have the aspects related to the waste deriving from the procedure been considered and have measures to decrease it been taken?
New Added Items to OPU ERA
If the animal or animals have already undergone the OPU procedure, were the procedure and the recovery of the animal carried out without difficulties?
Does the facility have an ethical internal committee?
Have measures/actions to avoid or minimise possible animal's injuries due to its partial control of the awareness during a) and b) been planned? a) pre-anaesthesia b) post-anaesthesia recovery
Have measures/actions to avoid or minimise any animal distress or suffering, during a) and b), been planned? a) pre-anaesthesia b) post-anaesthesia
Are measures/actions to avoid or minimise the potential negative influence of a), b) and c) on the welfare of the animal/s involved in the procedure been planned? a) Visual/olfactory/auditory inputs from other individuals b) Visual/olfactory/auditory absence of inputs from individual/s of the same social group c) Absence of familiar keeper/s.
Are measures/actions to avoid or minimise the potential negative influence of a), b) and c) on the welfare of other animal/s not directly involved in the procedure been planned? a) Visual/olfactory/auditory inputs from other individuals b) Visual/olfactory/auditory absence of inputs from individual/s of the same social group c) Absence of familiar keeper/s.
New Added Items to IVF-Lab EES
Have the aspects related to the environmental impact of the equipment and materials been considered and have measures to decrease it been taken?
Have the aspects related to the waste deriving from the procedure been considered and have measures to decrease it been taken?

2912

Discussion

The application of the ETHAS to the procedures performed during the present study both contributed to the overall acceptability of the project and improved communication among the projects' partners while refining the tool itself, in view of its standardization and application to other contexts in which ARTs are used for mammalian conservation projects.

Regarding the procedures assessed in the present study, it is important to note how having applied a tool which integrated risk assessment (general, ethical, welfare), pain/distress/welfare evaluation, harm–benefit analysis and the 3Rs tenet more likely had the potential to make the assessment and, eventually, help in the detection of problematic issues than using only one of these approaches separately. If we analyze, in more depth, the results of the ETHAS assessment, the harm–benefit analysis part allowed highlighting both positive effects and harms that could be generated by the execution of the OPU and IVF-lab procedures on wild animals and their specimens. Among positive effects highlighted during the assessment were: routine health and welfare check-up of the animals involved; the possibility of propagation of the genetic material of the specimens involved; scientific knowledge and know-how improvements that might find positive applications in other fields; the development of new technologies and procedures to promote the health and welfare of wild animals; the development of protocols for the conservation of endangered wild species. It was also possible to check whether the BioRescue team was committed to sharing the outcoming benefits with local communities. The restoration of the NWR's wild populations can directly positively affect local communities' economies through tourism and indirectly improve the quality of local communities' lives, restoring the African ecosystem and landscape [113,114]. The ETHAS confirmed that the know-how deriving from the procedures' optimization was shared with local veterinarians.

The local staff was also directly involved in the compilation of the OPU ERA, since a general and comprehensive goal of the ETHAS is to facilitate discussion among participants. The testing of the ETHAS confirmed that the tool was effective in this respect. As the results of the OPU ERA checklists showed, after the first application, the issues with negative answers caused by a

lack of communication were not detected in the second one. In general, better communication among participants helps to avoid, reduce or manage the risks of the procedures and to guarantee high standards. The application of the ETHAS to the laboratory procedures contributed to guarantee high standards also in the IVF procedure and to safeguard the biomaterial involved, as the three embryos created by NWRs are of exceptionally high conservation value.

Through the ETHAS, it was also possible to check for potential harms that may occur during the procedures and if everything possible was done to avoid their occurrence. The main potential harms highlighted by ETHAS application mostly concern the possible side effects of the veterinary procedures on the animals' health and welfare, correct preservation of the biomaterial and staff safety. However, since potential risks might occur during the veterinary procedures on wild animals, ETHAS application allowed highlighting the above-mentioned critical points, investigating whether action plans have been developed to deal with them and facilitating discussion around them between the staff members.

With regard to the animal welfare issues involved in the procedure, as highlighted by the positive results of the items specifically designed in the OPU ERA and EES, it was found that the team was committed to preserve and protect animal welfare, by monitoring the animals before, during and after the procedures, through physiological and behavioral analyses. Moreover, even if scientific evidence shows that the OPU procedure can be repeated on the same animal several times, the ETHAS allowed for checking if an adequate time-lapse between procedures was respected, as dictated by the best veterinary practices. Furthermore, specific items of the ERA checklist were included in order to analyze the welfare of other animals not directly involved in the procedures, such as herd mates sharing the same facilities or even enclosures.

Implementation of the 3Rs was another purpose of the ETHAS. Results showed that refinement, reduction and replacement were applied in the procedures whenever possible. For instance, refinement was applied by developing a new instrument for oocytes pick- up in rhinoceroses and by improving the procedures and techniques, with the aim of increasing the welfare of the animals involved, the efficacy of the procedures and the correct preservation of specimens.

Another aspect related to refinement was the inclusion of items regarding environmentally friendly waste disposal in the EES, after the first trial. The replacement of laboratory media with synthetic ones, the replacement of materials with lower environmental impact and the replacement of procedures and equipment with a lower impact on animal welfare were considered and applied whenever possible. Finally, reduction was implemented by maximizing the number of sampling procedures under the same anesthesia to reduce the number of veterinary interventions as much as possible.

Furthermore, the applications of the ETHAS in different conditions (zoos and semi- captive management) have contributed to refine the accuracy and inclusiveness of the tool itself. OPU and IVF-lab ERAs underwent several applications that allowed improving the tool via a shared work between ethicists and experts. This process permitted reviewing and refining the checklists iteratively through a participative approach.

Last, but not least, a general and comprehensive goal of the ETHAS was to assist scientists to carry out a self-assessment in addressing ethical evaluation of ART application in conservation projects. The results of the present study show that the application of such an ongoing assessment was effective in ensuring the high standards of the procedures, including respect for animal welfare, and facilitating effective communication among participants. It is important to note that the application of a form of ethical self-assessment to procedures or projects constitutes in itself a contribution to their acceptability even if no problematic issue is detected. All this is a value in itself and can increase acceptance of this kind of project by the public.

Limitations and Future Developments

Self-assessment can also be seen as the main limit of ETHAS application, as the evaluation process can be interpreted as self-referential. Nevertheless, as already pointed out, the primary function of ethical self-assessment is to help scientists think, in detail and proactively, through ethical issues surrounding their research. Usually, ethical evaluation regarding conservation projects, when it is performed, is made by an external authority, which gives a general ethical

approval to the overall project before it starts. On the contrary, ethical self-assessment offers the opportunity for an ongoing detailed scrutiny of all the main ethical aspects involved in the project, including the procedures that are carried out on animals, being proactive in detecting hazards for their welfare and taking measures to minimize them beforehand. In general, ethical self-assessment allows for a comprehensive and transparent evaluation process which can also be communicated to the public.

Another difficulty in applying such tool is the balancing between the need for standardization and that for customizing procedures and situations. Moreover, the fact that the tool is designed to evolve through iterative confrontation makes standardization more difficult. Notwithstanding, the ETHAS will continuously be tested in different contexts, species and procedures, in order to increase the comprehensiveness of the tool. However, it is important to note that the general frame and most of the tool are already adaptable to a more general use in different contexts, species and procedures, such as semen collection, embryo transfer, surrogate pregnancy and birth management, and to other innovative procedures regarding stem cell-associated techniques.

Conclusions

Ethical assessment of the application of ARTs in conservation is important for many reasons. In conservation breeding programs, for instance, animal welfare is a crucial element to be considered, alongside safety for the people involved and the quality of the procedures. Moreover, ethical assessment—especially when performed in the guise of self-assessment—allows anticipating the critical aspects that can compromise the ethical acceptability of a procedure and intervening before their eventual occurrence could damage the reputation of the whole conservation project and alienate societal support. As ARTs will become ever more important for conservation, the need to expand and deepen the ethical research on this topic will increase. An exemplary case, in this sense, is provided by the BioRescue project, which, alongside the development and testing of new approaches in the conservation of a “technically extinct” species, implemented a self-assessment tool designed for improving the procedures from an ethical

standpoint. The application of such a tool within the project allowed for the mutual goals of improving some aspects of the communication among the projects' partners and improving the tool itself, to be applied in the near future to other contexts in which ARTs are applied for the conservation of other mammal species. Despite the obvious advantages of this kind of self-assessment, such an approach is almost underestimated in the literature dealing with ART in conservation, as shown by a simple Scopus search on the subject. Therefore, tools such as the ETHAS could raise the ethical standards of applications of ARTs to conservation and, in this way, contribute to their success.

Supplementary Materials

The following are available online at https://www.mdpi.com/2076-261 5/11/2/312/s1, File S1: OPU EES_1st trial, File S2: OPU ERA_october2019, File S3: IVF-lab EES_1st trial, File S4: IVF-lab ERA.

Author Contributions

Conceptualization, B.d.M.; methodology, B.d.M., D.F., M.M.S., I.P., F.A. and S.N. (Simona Normando); formal analysis, I.P., M.M.S. and F.A.; investigation, I.P., M.M.S., D.F. and F.A.; data curation, I.P. and M.M.S.; writing—original draft preparation, I.P. and M.M.S.; writing—review and editing, P.B., S.N. (Simona Normando), D.F., F.A., S.S., C.G., G.L., S.C., T.B.H., F.G., R.H., S.H., J.Z., J.S., R.V., S.N. (Stephen Ngulu), S.M., and D.N.; visualization, I.P.; supervision, B.d.M., T.B.H. and S.N. (Simona Normando); project administration, B.d.M.; funding acquisition, T.B.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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1.3 The customization of the ethical assessment tool (ETHAS) for the assessment of advanced assisted reproductive technologies for in-vitro gametes of northern white rhinoceros (*Ceratotherium simum cottoni*)

Simply summary

Earth has entered an era of rapid biodiversity decline. Assisted reproduction technologies are becoming increasingly relevant in conservation wildlife projects. Recent advancements in mammal reproduction scientific knowledge led to the possibility of producing in-vitro gametes. This innovative reproductive biotechnology has been successfully performed only in murine models. However, it is promising, and its application to wildlife breeding programs will be very useful for projects aimed at the genetic restoration of critically endangered species. However, this reproductive technology still needs to be optimized, and its use in conservation projects has ethically significant consequences that need to be evaluated. The present work presents the customization and the first application of an ethical tool, ETHAS, to the laboratory procedures used to produce iPSCs from fibroblast of northern white rhinoceroses (*Ceratotherium simum cottoni*) and to the procedures that use these iPSCs to produce in-vitro gametes.

Abstract

Recent advancements in mammal reproduction technologies based on stem cells and in-vitro gametogenesis offer an unprecedented opportunity for conservation breeding projects. Pluripotent stem cells (PSCs), including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can be induced to complete the entire gametogenesis cycle in vitro. These advanced reproduction technologies (aARTs) allow conservationists to increase genetic variability in critically endangered species, reshuffling the chromosome through meiosis and even creating *ex novo* gametes of now-dead individuals. However, aARTs entail several ethical issues, and their use in conservation projects must be carefully evaluated in any phase of

their application. This work presents the customization of ETHAS, the ethical assessment tool for mammals' assisted reproduction technologies, to laboratory procedures used to produce induced pluripotent stem cells (iPSC) and in-vitro gametes. ETHAS is an ethical self-assessment tool that has been developed by the Ethical team of the BioRescue consortium. BioRescue is formed by an international team of scientists of different disciplines that are using assisted reproduction technologies (ARTs) with natural gametes to produce embryos of the northern white rhinoceroses and are developing aARTs to produce in vitro gametes to save this iconic animal from extinction. ETHAS consists of two checklists, the Ethical Evaluation Sheet and the Ethical Risk Assessment, and it provides an integrated, multilevel, and standardized self-assessment of the procedure under scrutiny. The first application of ETHAS to two laboratories for advanced reproductive technologies, one in Japan and one in Germany, showed that the tool was able to highlight some critical issues arising from the use of these procedures and promoted communication among the project's partners. The tool underwent a revision process through an iterative consultation process between experts (both ethicists and scientists), and its implementation will proceed following the advancement in the development of the procedures of northern white rhinoceroses in-vitro gametogenesis.

Introduction

Earth has entered an era of rapid biodiversity decline. Many species have very limited, fragmented populations in the wild. For species with small population size, isolated populations, and low reproductive success, breeding programs play an increasingly crucial role in preserving genetic diversity (Herrick et al., 2019; Comizzoli et al., 2019). Wildlife conservation breeding projects can be enhanced with the use of assisted reproduction technologies. Assisted reproduction technologies can be defined as any procedure or technique that involves the handling of gametes or embryos to achieve reproduction and improve a species' genetic management (Ebenhard 1995; Comizzoli 2015; Herrick 2019). Classical reproduction assisted

technologies (ARTs) — such as assisted insemination (AI) or in-vitro fertilization (IVF) — use natural gametes to increase the probability of generating newborns.

However, for some species with already too few individuals alive, ART can no longer be sufficient to maintain genetic diversity for the population's long-term sustainability. For this species, advanced assisted reproduction technologies (aART), which use somatic cells to generate in-vitro gametes, might represent the last resort to be saved. ART and aART increase the chances of successful conservative breeding programs by overcoming infertility and optimizing genetic management, avoiding inbreeding (or outbreeding) depression, and risks of inherited disease transmission.

However, aART, in particular, represents an innovative approach to conservation and has ethically significant consequences for conservation projects, people, animals involved, and ecosystems, which should be carefully discussed (Biasetti et al., 2022). The use of aARTs in conservation projects must be well justified and the objectives clearly defined, and an evaluation of the project's success in terms of feasibility and ecological, social, and scientific values must be performed (Sandler et al., 2021). Additionally, an ethical evaluation of every single procedure should be made at every stage, from the planning of the procedure to its implementation and its outcome.

In this work, it is presented the customization of the Ethical Assessment Tool (ETHAS) to evaluate the procedures used for generating iPSCs and in-vitro gametes of northern white rhinoceroses. Despite the increasing use of ART and the initial use of aART in endangered species breeding programs, a dedicated framework for evaluating their application is still missing. A lack of attention to ethical evaluation can be seriously detrimental to the welfare and lives of the animals involved, the newborns, the quality of research, the safety of personnel involved, the fairness of benefit sharing, and, more generally, the success of conservation projects. ETHAS Assessment Tool has already been used to assess the procedures for collecting ova from the females of northern white rhinoceroses (*Ceratotherium simum cottoni*) (de Mori et al., 2021).

The tool was developed to assess the procedures of aARTs implemented by the scientists of the BioRescue consortium in the attempt to save the "technically extinct" Northern white rhinoceroses.

The BioRescue consortium was founded by the German Federal Ministry of Education and Research (BMBF), led by the Leibniz Institute for Zoo and Wildlife Research (Leibniz-IZW), and composed by Czech Dvůr Králové Zoo, Avantea srl, Max Delbrück Center for Molecular Medicine (MDC), Kyushu University and Padova University.

The scientists of BioRescue proposed an innovative approach that combines the use of natural gametes and in-vitro gametes to save the northern white rhinoceros (*Ceratotherium simum cottoni*) from extinction. Natural gametes can be obtained by ovum pick-up from living females and cryopreserved sperm of now-dead males. Specimens of sperm from five different individuals are cryopreserved in biobanks in addition to the frozen testicular tissue of two individuals that could be used in the future for in-vitro gametogenesis (Saragusty et al., 2016). No oocytes are stored in biobanks because of their low permeability to cryoprotectants and consequent susceptibility to frozen (Woods et al., 2004). However, frozen tissues of fourteen individuals, nine females and five males, are stored (Saragusty et al., 2016). The somatic cells of these tissues can be used to create induced pluripotent stem cells (iPCS) that can produce in-vitro gametes through the in-vitro gametogenesis process. The gametes can be used for in vitro fertilization (IVF), and the embryos, after maturation, can be cryopreserved or transferred into recipient mothers of a related species, such as the southern white rhinoceros (*Ceratotherium simum simum*), that will give birth to northern white rhinoceros newborns.

The scientists of Avantea srl, Max Delbrück Center for Molecular Medicine (MDC), and Kyushu University are currently working on the laboratory procedures for assisted reproduction of the northern white rhinoceroses, as members of the BioRescue consortium. Specifically, the scientists of Avantea srl are performing the in vitro IVF procedures with natural gametes, the scientists of the Department of Stem Cell Biology of the Max Delbrück Center are

reprogramming somatic cells of rhinoceroses into iPSCs, and the scientists of the Department of Basic Medicine of the Kyushu University the in-vitro gametogenesis process.

Although assisted reproductive technologies, ARTs, that use natural gametes are already widely applied to laboratory and farm animals, their use in wildlife is less established. On the other hand, advanced assisted reproductive technologies, aARTs, that use in-vitro gametes ARTs have so far only been successfully applied to mice, whose reproductive biology is well known and often less challenging. However, using aARTs to perform in-vitro gametogenesis, thanks to meiosis, can enhance critically endangered species' genetic management and restoration. Indeed, this approach can create an enormous variety of new genotypes by reshuffling existing diversity with the crossing-over that generates new chromosome reassortments.

However, the use of the procedures of aART is still at a very early stage of optimization and entails a certain grade of uncertainty that open up new ethical scenarios that require ethical analysis at all stages of these procedures. This is critical because ethically weak procedures risk compromising the ethical acceptability of projects with otherwise commendable goals.

In-vitro gametogenesis

To produce in-vitro gametes from somatic cells, such as fibroblasts, scientists use stem-associated technologies (SCATs). The somatic cells can be reprogrammed into induced pluripotent stem cells (iPSC) that, similarly to embryonic stem cells (ESCs), can grow indefinitely while maintaining pluripotency and are capable of differentiating into all three germ layers (Takahashi & Yamanaka, 2006). However, unlike ESCs, iPSCs do not require embryonic tissues for harvesting. The iPSC has been successfully established for several domestic and laboratory species, but also for endangered species, such as the snow leopard, and critically endangered species, such as the northern white rhinoceros (Bank et al., 2021; Friedrich Ben-Nun et al., 2011; Korody et al., 2021; Verma et al., 2012; Zywitza et al., 2022).

The most widely applied approach to creating iPSC lines is to use Yamanaka reprogramming factors OCT4, SOX2, KLF4, and cMYC (Stanton et al., 2019; Takahashi & Yamanaka, 2006). Further research on species-specific reprogramming factors is needed to overcome the difficulties in producing stable, transgene-free iPSCs (Stanton et al., 2019). However, recently, improved and reproducible methods have been applied to produce iPSCs from northern white rhinoceros cells that allow the production of transgene-free iPSCs, that do not require a continued expression of exogenous reprogramming factors to maintain pluripotency, and that, therefore, can be used for gametogenesis in vitro (Korody et al., 2021; Zywitza et al., 2022).

Given their pluripotency, induced pluripotent stem cells can differentiate into all different embryo tissues, including the germ cell lineage, and, under appropriate conditions recreated in vitro, the germ cell lineage can mature into gametes. This procedure is called in vitro-gametogenesis. All germ cell lineages originate from primordial germ cells (PGCs), which are segregated from the somatic cell lineage at an early developmental stage when a characteristic gene expression program appending genome-wide epigenetic change is observed in epiblast cells heading to PGCs (Hayashi & Saitou, 2014).

The research in murine models shows that the germ cell lineage is derived from the pluripotent cell population in response to extrinsic signals. Evidence from genetic studies has uncovered the extrinsic signals essential for PGC specification, and researchers think they could also be used for other mammals (Hayashi & Saitou, 2014). However, there are distinct types of pluripotent states with respect to the responsiveness to extrinsic signals. Studies in vivo have revealed that it is likely that ESCs acquire PGC-competence during conversion from the naïve to primed pluripotent state (Hayashi & Saitou, 2014). Therefore, the induced pluripotent stem cell to be used in in vitro gametogenesis must reach the naïve pluripotent state (Hayashi & Saitou, 2014; Zywitza et al., 2022). The following step is the reconstruction in vitro of the PGCs specification processes to convert the naïve state to an epiblast-like state with PGC-competence, the PGC-like cells (PGCLCs), under a defined set of conditions and extrinsic signals (Hayashi et al., 2011). The PGCLCs have a similar pattern of gene expression and genome-wide reorganization of

epigenetic modification similar to that of PGCs in vivo (Hayashi & Saitou, 2014). The final step is represented by the differentiation of PGCLCs into spermatozoa and oocytes. The process in vivo depends on the environment of the gonads, testis, and ovary, respectively (Hayashi & Saitou, 2014). In vitro, the environment required for the sexual differentiation of PGCLCs in germ cells, is achieved by co-culture of PGCLCs with embryonic gonadal somatic cells (Hikabe et al., 2016; Ishikura et al., 2016). However, for in-vitro gametogenesis in the context of endangered species, the fetal gonadal somatic must be very difficult to obtain. In these cases, xeno-reconstituted ovaries with mouse fetal gonadal somatic cells would be one option to bypass this obstacle (Hayashi et al., 2021). Although partially positive results have been reported for the in vitro generation of germ cells in several mammalian livestock species, currently, mice remain the only species for which germ cell development has been fully reconstituted in vitro (Hayashi et al., 2017; Hikabe et al., 2016; Ishikura et al., 2016).

Method

The prototype of ETHAS for laboratory procedures had already been created and tested to assess IVF procedures with natural gametes of northern white rhinoceroses performed in the laboratory of Avantea srl. The results had been published in de Mori et al., 2021, and can be found in the present work in section 3.1.

The methodology and the frame developed, in the previous work, for the IVF laboratory were applied to create a customized ETHAS for the procedures of iPSCs and in-vitro gametogenesis. The tool was then tested on the procedures performed in the Department of Stem Cell Biology of the Max Delbrück Center and in the Department of Basic Medicine of Kyushu University.

The customization of the tool was done after a review of the most recent scientific literature on aARTs, legislation, international treaties, and ethics. Its development was also based on best laboratory practices and guidelines and refined through an iterative consultation process between experts (both ethicists and scientists).

The tool (ETHAS)

In this work, we used the frame of ETHAS described in de Mori et al., 2021. Hereafter, it is shortly described highlighting the customization for the laboratory procedures for iPSCs and in-invitro gametes production.

ETHAS is a flexible and customizable tool that scientists can use for an ethical self-evaluation, in this case, for the specific advanced laboratory procedure of aARTs they are planning to perform. The tool is based on checklists, a valuable tool for self-assessment to identify errors and check the conformity to operational standards, best practices, ethical tenets (such as 3Rs), and normative. The self-assessment tools help scientists to be proactive and examine the ethical issues surrounding their work, and make them easier to be communicated, discussed, and addressed; and, in doing so, it contributes to the responsible conduct of research, thereby increasing its public acceptance (Horizon 2020).

The general frame of the ETHAS tool is based on two integrated checklists for self-assessment, the Ethical Evaluation Sheet (EES) and the Ethical Risk Assessment (ERA). ETHAS's checklists were developed with the aim of combining the risk assessment of the specific laboratory procedures with the ethical acceptability assessment. To this aim, each ERA item is conceptually linked to a corresponding part of the EES checklist, which comprises, among others, all the relevant ethical aspects that are investigated in ERA. The link is reported in a column with an alphanumeric code. EES is conceived to be filled in once at the beginning of the project to assess the design of the procedure. On the other hand, ERA is conceived to be filled each time the laboratory procedure is performed during its optimization or whenever there are changes in the protocol, and just once, at the beginning of the project, for optimized and standardized procedures.

Ethical Evaluation Sheet (EES)

The Ethical Evaluation Sheet (EES) checklist contains items that highlight potential ethical issues arising from the procedures IPCs production and in-vitro gametogenesis applied to mammal conservation projects (Attachment 1). It was developed by analyzing corresponding tools for the ethical assessment of research projects with laboratory animals (Horizon 2020; Smith et al., 2007). EES consists of four main sections of investigation: Documents; Harm–benefit evaluation, Procedure quality evaluation, and Scientific team quality evaluation.

The section on "Documents" checks compliance with the relevant regulations and laws (i.e., Nagoya protocol, CITES, etc.), as well as with best practices. The section "Harm and benefit evaluation" analyses the procedure for the project, tracking its ethically relevant consequences, including possible consequences for the specimens, the newborns, the species, the biodiversity conservation, the environment, the scientific and technological advancement, and people and communities involved. Procedure quality evaluation measures the degree of robustness of the scientific and technological background of the procedure, as well as compliance with the 3Rs. Finally, scientific team quality evaluation assesses the degree of experience and coordination of the team performing the procedure, the satisfaction with ethical research standards, and the quality of the equipment and laboratory and biobank evaluation.

Table 1 reports the number of items and subitems for each section and the bibliography consulted to develop Biomolecular laboratory EES.

EES Sections and Sub-Sections	Number of items (sub-items)	Bibliography
(A) Documents	8 (8)	Bout et al., 2014; Horizon 2020; Directive 2010/63/EU; Cartagena Protocol; Nagoya Protocol; Sherkow et al., 2022; J. A. Smith et al., 2007
(B)Harm–benefit evaluation (B1) Benefit evaluation (B2) Harm evaluation	8 (8) 5 (10)	Bout et al., 2014; Brønstad et al., 2016; Huges et al. 2010; Hooijmans et al., 2010; Kilkenny et al., 2010; Percie Du Sert et al., 2020; Smith et al., 2018
(C) Procedure quality evaluation **(**C1) Pre-screening consideration (C2) Procedural steps evaluation	6 (10) 5 (5)	Directive 2010/63/EU; Redford et al., 2019
(C3) 3Rs evaluation (replacement, reduction, refinement)	12 (23)	De Mori, 2019; Hooijmans et al., 2010
(D) Scientific team quality evaluation (D1) Team and teamwork (D2) Equipment (D3) Laboratories and biobanks	11 (17) 3 (3) 3 (3)	De Blasio & Biunno, 2021; Dolan, 1999; ESHRE, 2015; A. J. Smith et al., 2018; Sherkow et al., 2022; OIE, 2018
(E) Final ethical evaluation of the procedure	10 (10)	(Dolan, 1999)

Table 1. Ethical Evaluation Sheet sections and bibliography

In the present work, the EES was filled in by a member of the BioRescue team with an ethical background. However, this was a phase of development of the tool, and in its final version, it will comprise the items of the two checklists into one, and any scientist performing ARTs procedures will be able to answer the items on the checklist.

During the EES compilation, it is asked to answer “yes” or “no” to all items, depending on whether the requirements are met or not. Moreover, for some EES items, it is required to add further information to explain the answer. The EES is evaluated using a semi-quantitative scoring model in which the answers “yes” or “no” assume the value of 0 and 1, respectively. The sum of the items’ outcomes divided into three homogeneous ranges defines the rank of the ethical acceptability of the procedure: not acceptable, partially acceptable, and acceptable. Therefore, the final score obtained from the EES compilation identifies one of the three acceptability ranks (Table 2).

Acceptability ranks	**Biomolecular-Lab EES**
Totally acceptable	0–28
Partially acceptable	29–56
Not acceptable	57–87

Table 2. Acceptability ranks for the Biomolecualar-lab procedures and biomolecular procedures applied ot the generation of iPCS and in-vitro gametes.

The identified acceptability level that represents the outcome of the EES assessment (defined as the first review level) defines the degree of the procedure’s acceptability. In case of a partial or not acceptable result in the ethical assessment, detected with the first review level, each section of the EES checklist is assessed individually. This second review level identifies at which section of the procedure corrective actions need to be planned. Finally, a third review level allows for identifying the items whose requirement is not met and, therefore, the critical issues of the procedure to be reviewed before the procedure begins.

Ethical Risk Assessment (ERA)

Applications of aART require the manipulation of live animals, the donors from which the specimens are collected, and cells in laboratory procedures. These manipulations may result in risks to the animals' welfare, the valuable biomaterials of the endangered species, the newborns, and, potentially, the whole species. For these reasons, it is necessary to evaluate the level of risk, and its acceptability must be assessed.

The ERA checklist is specifically customized for the risk assessment of each phase of the procedure.

For the development of this checklist, the scientific literature on the procedures for the generation of iPSC and invitro gametes have been revised to detect possible hazards and ethical risks that could negatively impact the biomaterials and the future newborns, staff safety, and the ethical acceptability of these procedures. Table 3 reports the number of items and subitems for each section and the bibliography consulted to develop the Biomolecular Lab ERA checklist (Attachments 2).

At the beginning of ERA, there is a preliminary section named "General risks - Possible benefits". This section contains items to evaluate if the potential benefits overcome the general risks. If this is not the case, researchers must stop and discuss with other project partners to address the potential issues.

Biomolecular-lab ERA Sections and Sub-Sections	Number of items (sub-items)	Bibliography
General risks– Possibile Benefits	5(5)	Griffin et al., 2014; Bout et al., 2014; Grimm et al., 2019; Gutfreund et al., 2020; Brønstad et al., 2016; Shwiff et al., 2013, Pulling et al.; 2019
(A) Laboratory quality assessment and specimens processing	41 (65)	De Blasio & Biunno, 2021; ESHRE, 2015
B) Specimens shipping to the Laboratory	8 (9)	ESHRE, 2015; Galli et al., 2016; OIE, 2018
(C) Specimens biobanking	10 (12)	Comizzoli et al., 2022; De Blasio & Biunno, 2021; ESHRE, 2015; Holt & Comizzoli, 2021; Parnpai et al., 2016; Portas et al., 2009; Prieto et al., 2014; Saragusty et al., 2020; Saragusty & Arav, 2011
(D) Induced Pluripotent stem cells (iPSCs)	14 (19)	ESHRE, 2015; Hildebrandt et al., 2018
(E) In vitro gametogenesis (IVG) *(E1) In vitro oogenesis* *(E2) In vitro spermatogenesis*	17 (25) 15 (21)	ESHRE, 2015; Galli et al., 2014; Vanden Meerschaut et al., 2014
(F) In vitro fertilization	9 (14)	ESHRE, 2015; Galli et al., 2014; Vanden Meerschaut et al., 2014
(G) Embryos culture	14 (16)	ESHRE, 2015; T. Hildebrandt et al., 2018; Vanden Meerschaut et al., 2014
(H) iPSCs, gametes, and embryos packaging	4 (7)	ESHRE, 2015; OIE, 2019
(I) iPSCs, gametes, and embryos shipping	7 (8)	ESHRE, 2015; OIE, 2018

Table 4. Biomolecular lab Ethical Risk Assessment sections and bibliography

The assessment uses a semi-quantitative scoring model where the risk is determined by a single value R that combines the probabilities (p) and consequences (x) associated with the occurrence of a hazard scenario (Kaplan et al., 1981). The hazard scenario is identified with each ERA item. The probabilities are determined by the satisfaction or not of the item. The consequences depend

on the characteristics of the requirement of the item and are classified into different levels of severity, in accordance with Table 5.

The risk is calculated as follows:

$$R = \sum_{i=1}^{n} p_i x_i$$

In the specific model, n corresponds to the number of scenarios chosen to describe the risk (number of items of the ERA checklist), p_i can assume values of 0 or 1 depending on whether the requirement is met (yes) or not (no/no answer) and x_i is from 1 to 3, as described in Table 5.

Level of consequences	**Characteristics of the Requirement**	**Score**
Low	Factors affecting the process (documental and procedural support aspects such as operating protocols)	**1**
Medium	Factors related to the traceability and distribution of specimens, laboratory operator's safety, quality and availability of laboratory facilities and instruments	**2**
High	Factors related to the viability of specimens, the instrumental requirements, and the chemical reagents used.	**3**

Table 5. Description of risk categories and corresponding score

As laboratory procedures use standardized methodologies, ERA checklist for laboratory procedures was designed to be filled in the first time the procedure is applied. However, if they are modified, a new evaluation with ERA must be performed. To evaluate the category risk of the procedure, the sum of the score of the item is divided into three ranges corresponding to the three risk categories (Table 6)

Risk rank	The score for the procedures to produce iPSC in Biomolecular lab-ERA	The score for the procedures to produce in-vitro gametes in Biomolecular lab-ERA
Low	0-76	0-65
Medium	77-152	66-129
High	153-229	130-194

Table 6. Risk ranks of IVF-lab Biomolecular lab-ERA

Similarly to the EES, also for the ERA, the assessment can be done at three levels: at the item level, at the phase level, or at the overall procedure level. At the item level, the assessment allows highlighting a specific risk; at the phase level, it allows highlighting the risk of that part of the procedure; and, at the procedure level, it allows highlighting the risk of using that specific procedure.

Final Overall Evaluation of ETHAS

The combination of the results of ERA and EES generates the final outcome of ERA and gives the overall evaluation of the procedure (table 7).

ERA / EES	Low Risk	Medium Risk	High Risk
Totally acceptable	Acceptable	Acceptable with mitigation	Not acceptable
Partially acceptable	Acceptable with mitigation	Acceptable with mitigation	Not acceptable
Not acceptable	Not acceptable	Not acceptable	Not acceptable

Table 7. Ethical Assessment Tool (ETHAS) overall final evaluation, obtained by combining results from the ESS and ERA checklists.

ETHAS's final evaluation of the procedure falls in three categories:

1. Acceptable: when the ESS results in totally acceptable and the ERA detects low risks. The assessed procedure may be accepted without further action.

2. Acceptable with mitigation: when the EES results in partially acceptable and the ERA detects medium risks. The assessed procedure may be accepted only if critical issues are identified and addressed, and the specific application of the procedure is revised.

3. Not acceptable: when the EES detects a not acceptable result, and the ERA detects high risks. The assessed procedure may be unacceptable until further improvements are enforced to eliminate the associated ethical concerns and procedural risks.

Application of the tool

The first version of ETHAS for the assessment of laboratory procedures for aARTs was prepared after consulting the relevant scientific literature and best practice guidelines on biomolecular laboratory procedures. However, as aART is an innovative approach to wildlife conservation, relevant aspects not found in the literature were discussed, by email or with video callings, with the scientists that are developing these procedures for northern white rhinoceroses. Additionally, the draft was also discussed with the scientists of Avantea with whom the previous ETHAS for IVF laboratory procedures was optimized and tested.

After the optimization, in March 2022, the tool was sent by email to the director and researchers of del Department of Stem Cell Biology del Max Delbrück Center for Molecular Medicin, Berlin, Germany, who are working to create induced pluripotent stem cells (iPSCs) from northern white rhinoceroses, and to the director of the Department of Basic Medicine of Kyushu University, Japan, who has successfully performed the in-vitro gametogenesis of mice gametes and is working on the in-vitro gametogenesis of gametes of rhinoceroses. They compiled the checklist and resent it to the researchers of the Ethical team of BioRescue.

The EES, in this preliminary phase, was filled in by researchers of the Ethical team.

RESULTS

EES results

	Positive answers	Negative answers
A) Documents	6 over 8	2 over 8
B) Harm–Benefit Evaluation	12 over 18	6 over 18
C) Procedure Quality Evaluation	23 over 38	15 over 38
D) Scientific team quality evaluation	22 over 23	1 over 23
TOTAL	63 over 87	24 over 87

Table 8. EES results for the laboratory biomolecular procedures applied to produce IPCS and in-vitro gametes.

The biomolecular-lab EES outcome was 24 over 87, and it assessed that these procedures are "Totally acceptable," although it is at the lower end of the range.

In the document section, the items that received a negative score were related to the fact that the laboratory did not have any accreditation (e.g., ISO, etc.) nor an internal ethical committee.

Regarding the laboratory procedures, the items asking if the application of this procedure was totally safe and with no adverse side effects on the specimens or the newborns received negative answers.

Additionally, the procedure quality evaluation received negative responses because not all the protocols had been optimized for rhinoceroses. Finally, the scientific quality section, an item received a negative score because it was the first time that the scientists of one of the laboratories were performing these procedures.

ERA results

The responses of the ERA checklists obtained from the scientists of the two laboratories located at the Department of Basic Medicine of Kyushu University, Japan, at the Department of Stem

Cell Biology del Max Delbrück Center for Molecular Medicine, Berlin, Germany were entered in Excel and compared. Both laboratories can perform the procedures to produce iPSCs, so the scientists of both laboratories answered the items of this section in the ERA checklist.

However, only the researchers in the Japanese laboratory have the know-how to perform the procedures for the in-vitro gametogenesis, so they were the only ones that answered to the items in this section. This aspect was anticipated during the development of the tool. At the beginning of each section, in the ERA checklist, respondents are asked whether or not the procedure to which that section refers is performed in the laboratory or project, and if the answer is negative, they are asked to skip the section.

In table 9 are shown the results of Biomolecular-Lab ERA.

	Laboratory n.1		**Laboratory n.2**	
	Positive answers	**Negative answers**	**Positive answers**	**Negative answers**
(A) Laboratory quality assessment and specimens processing	40 over 79	39 over 79	37 over 79	42 over 79
B) Specimens shipping to Laboratory	9 over 13	4 over 13	8 over 13	5 over 13
(C) Specimens biobanking	15 over 38	23 over 38	29 over 38	9 over 38
(D) Induced Pluripotent stem cells (iPSCs)	46 over 99	53 over 99	65 over 99	34 over 99
TOTAL	110 over 229	117 over 229	139 over 229	90 over 229

Table 9. Biomolecular-Lab ERA for assessing the procedures to obtain the iPSC. Laboratory n.1 is the located at the Department of Stem Cell Biology del Max Delbrück Center for Molecular Medicine, Berlin, Germany. Laboratory n.2 is the located at the Department of Basic Medicine of Kyushu University, Japan.

	Laboratory n.2	
	Positive answers	**Negative answers**
(E1) In vitro oogenesis **(E2) In vitro spermatogenesis**	20 over 64 18 over 64	9over 64 17 over 64
(F) In vitro fertilization	31 over 57	26 over 57
(G) Embryos culture	24 over 32	8 over 32
(H) iPSCs, gametes, and embryos packaging	13 over 27	14 over 27
(I) iPSCs, gametes, and embryos shipping	7 over 14	7 over 14
TOTAL	113 over 194	81 over 194

Table 10. Biomolecular-Lab ERA for assessing the procedures for in-vitro gametogenesis in the located at the Department of Basic Medicine of Kyushu University, Japan.

The assessment of the iPSCs in both laboratories resulted in a medium risk. The first level of the analysis shows a high variability of responses between the two laboratories. The first aspect highlighted by the ERA results is that the responses between the two laboratories varied considerably.

For example, the two laboratories had different procedures for sample handling. Whereas in Germany, incoming samples were opened in secure cabinets, quality tested, and access to the biobank and sample handling times were recorded in a database, this was not the case in Japan. Additionally, many items received negative responses because these procedures had never been applied to cells of rhinoceroses before. Therefore, all items asking whether the cellular processes for iPSC production were known and had been evaluated in murine models received positive responses but negative responses when referring to the rhinoceros. The same applies when asked whether possible epigenetic changes are known and have been evaluated. However, both laboratories answered that they use standardized protocols to evaluate the iPCSs produced before

using them for in-vitro gametogenesis and identify the presence of genetic abnormalities or exogenous DNA sequences of the construct used for their production. All differing responses were discussed with the researcher who performed the iPSC procedures for the northern white rhinoceroses in Germany and who will work on the in-vitro gametogenesis in the laboratories in Japan as visiting researcher.

The procedures of in-vitro gametogenesis are at a very early stage of optimization; therefore, only the items regarding the murine model received positive responses.

The following items received a positive answer:

a) Since for a complete in vitro spermatogenesis, it is necessary a tight interaction between spermatozoa and gonadal cells (Sertoli cells), is it possible to use an in vitro culture of testicular tissue to recreate the testis environment?

b) Since for a complete in vitro oogenesis it is necessary, especially in the latter phases (meiosis, follicle formation, and oocyte growth), tight interactions between oocytes and gonadal somatic cells, is it possible to use reconstituted ovary (rOvary) to recreate the ovarian environment?

These responses show that these procedures can be carried out without using in-vivo procedures. However, the items that were investigating the possibility of using laboratory or farm animals to reconstitute the gonads' natural environment for the development of in-vitro gametogenesis of the endangered species received negative responses. This aspect must be taken into consideration for the feasibility of using these procedures in conservation because it is often difficult to access biomaterials of endangered species.

The combination of the results of ERA (Low risk) and EES (Totally acceptable) for the procedures allowed us to assess these procedures as Acceptable for the BioRescue project conservation of the northern white rhinoceroses.

For the procedure applied to the production of iPSC and in-vitro gametes, the combination of the results of ERA (Medium risk) and EES (Totally acceptable) allowed us to assess these procedures as "Acceptable with mitigation". Therefore, they can be used in the conservation project of BioRescue, but they require further optimizations.

Discussion

The results of the application of ETHAS to the assessment of the first phase of the production of in-vitro gametes from somatic cells, i.e. the procedures for the production of iPCS, showed that the application of these procedures on rhinoceroses biomaterials are acceptable, under the conditions which they were performed by the scientists of BioRescue consortium, because even if they entail some risks for the biomaterials, highlighted by the single items of the ERA, the scientific knowledge achievable is relevant for wildlife conservation. Additionally, the risk level for the biomaterial was considered not relevant because for the optimization of the procedure, it was decided to use specimens of a northern white rhinoceros, Nabire, that displays aneuploidy and will not be used for generating in vitro gametes (Zywitza et al., 2022). However, the results of ERA showed that there was great variability in terms of laboratory management, specimen processing, and reproducibility of the procedures between the laboratory evaluations. The results obtained were discussed with the participants in order to reach a higher standardization of the procedures.

However, the laboratories evaluated are research laboratories and do not necessarily have the same standardization required in a private laboratory that offers services to the public, such is the case of Avantea srl, where the ERA for the in vitro fertilization was tested. Avantea srl has the Quality Certification UNI EN ISO 9001:2015 that requires the standardization of all the procedures, laboratory management, etc.

For what concern the section of the tool that assesses the procedure of in-vitro gametogenesis, as these procedures for rhinoceros' cells are still at a very early stage, the tool was tested on the procedures of in-vitro gametogenesis in murine models with the researcher that have already produced available in-vitro gametes of mice (Hayashi et al., 2018). Nonetheless, the results of this application were relevant for the implementation of the tool.

The in-vitro gametogenesis associated with stem cell technologies represents an innovative tool in reproduction technologies. These technologies can boost the genetic variability of species on the verge of extinction. However, they entail several ethical issues, and their application to

conservation projects must be assessed with an ethical analysis throughout all the phases of the project. The ethical analysis must evaluate the quality of these procedures and their compliance with the current legislation and the best practices in the field. Next, it must assess the potential benefits deriving from safeguarding biodiversity, the possible positive social consequences, the scientific and technological advancements that the application of these technologies can achieve, and whether they are carried out in a responsible and sustainable way. The ethical analysis permits us to determine whether a procedure is acceptable according to specific standards of value and to identify the critical issues that need to be addressed before its implementation. ETHAS can help scientists in all these phases of the evaluation of their project.

Future developments

Every researcher should be able to make an ethical assessment of the procedures he or she is using both in the design phase and in the phase prior to the application of the procedure. However, not all scientists have an ethical background that can help them in this assessment. In these cases, it is valuable to have a self -assessment ethical tools that can help with this task. For this reason, the future development of ETHAS will create a friendlier easier-to-use version of the tool. The new tool, now under development, will include items from the ERA and EES in a single checklist. All items repeated in ERA and EES, because they had to be evaluated by both the respondents, will be eliminated and some items will be reformulated. In the future tool, the items considered essential to be satisfied for the ethical acceptability of the procedure will be marked with a red dot so that the researcher can immediately see if the procedure needs implementation. The results of the tool will be visually evaluated as the person who fills the checklist will simultaneously mark a dot in the corresponding section of a table. In this way, the researcher can quickly see if there is part of the procedure (identified by a section of the checklist) he or she needs to improve in order to make the procedure acceptable.

Conclusions

Currently, all five surviving rhino species are classified as endangered or critically endangered, and illegal hunting for their horns remains the most severe threat (Gross, 2018).

aART represents a powerful tool in the toolkit of conservationists to halt and revert the loss of biodiversity. The innovative approach proposed by BioRescue scientists for the northern white rhinoceros, once optimized, can be applied to other rhinoceroses' species. Furthermore, with appropriate species-specific adaptations, this approach can be applied to all mammal species on the verge of extinction.

Because the laboratory procedures for aARTs are innovative and still require optimization, they entail a certain level of degree of risk. The evaluation of the level of risk of these procedures should be done using the "as low as reasonable applicable principle (ALARP)" together with the Precautionary Principle (Ersdal & Aven, 2008). The risk assessment of each phase of the procedure can prove if it can be considered "reasonably safe" (Tickner et al., 2003). In this way, the Precautionary Principle provides a certain degree of operativity for any research aiming to design new conservation strategies, even if there is a certain level of unpredictability. This unpredictability can be ethically acceptable only when a risk assessment is performed on the procedures to highlight potential risks and evaluate them in terms of occurrence and outcome, plan mitigation actions, and evaluate possible alternatives. In this way, even if the risk probability is never zero, it can be taken to a tolerable threshold level. That is the reason why the risk assessment must be integrated into the ethical analysis.

ETHAS can help scientists in the ethical self-assessment of the advanced reproductive procedures they are planning to use in their conservation projects as it integrates the risk assessment in the ethical self-assessment and allows for assessing potential risks continuously and in advance.

Additionally, the tool can contribute to helping scientists in thinking ethically about their work, address potential issues, and clearly communicate them. Open and transparent communication and the demonstration that the projects are conducted with the highest ethical and scientific

standards will help the public to understand the potential risks and, at the same time, accept a certain grade of risks if well justified. Only in this way the researchers and conservationists will have the trust and support of the public, even if the precautionary principle is not applied in its "strong" formulation, which would call for absolute proof of safety (Carolan, 2007). Applying the precautionary principle in its "strong" formulation, especially to aART, could preclude using these assisted reproduction technologies, and many species could be condemned to extinction. Indeed, after optimization, these biotechnologies will be essential, together with in-situ and classical ex-situ conservation projects, to save many endangered species on the verge of extinction. Then, conservationists will be able to have more tools in their toolbox to safeguard the "infinite most beautiful and most wonderful forms" (Darwin, 1859) that have so far shared the Earth with humans.

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SECTION 2

ETHICAL ANALYSIS OF NEW CHALLENGES IN WILDLIFE CONSERVATION

2.1 Development of A Tool for Assessing the Ethical Reputation of Zoos:

The Zoo Ethical Reputation Survey (ZERS)

This chapter is adapted from:

Spiriti, Maria Michela, Francesco Maria Melchiori, Paul Wilhelm Dierkes, Linda Ferrante, Francesca Bandoli, Pierfrancesco Biasetti, and Barbara de Mori. "Development of A Tool for Assessing the Reputation of Zoos: The Zoo Ethical Reputation Survey (ZERS). Animals 12, no. 20 (2022): 2802.

Simple Summary

The reputation of a zoo indicates the level of public consideration of this institution and is determined by the actions, values, and behaviors that it has conveyed over time. The reputation of zoos is a complex construct and highlighting the key factors that can negatively affect it can lead to identifying ways to promote their reputation. To address these critical issues, a zoo must not only promote higher operational and ethical standards and animal welfare but also be certain that the stakeholders perceive the importance of its mission. This will benefit the individual institution and zoological institutions as a whole as a positive reputation will enable zoos to thrive in the future as biodiversity conservation institutions and places of environmental education and entertainment publicly supported. In this work, we report the development and the first trial of the Zoo Ethical Reputation Survey (ZERS), a tool that, through a survey designed with ad hoc items, analyzes public opinion on features that can influence the reputation of a zoo, focusing on ethical aspects. During its first applications, ZERS proved to be a tool able to provide information on the visitors' opinions about several drivers that, according to the literature, influence corporate reputation.

Abstract

Nowadays, most zoos have taken prominent and active positions in endangered species conservation and educating visitors about the value of biodiversity. However, to be effective and trusted in their mission, they must act ethically and have a good reputation. Yet, the drivers that can influence their reputation are still little investigated, and there are still few studies focused on assessing the reputation of these institutions. In the present work, we report the development of a tool, the Zoo Ethical Reputation Survey (ZERS), and its pilot application to assess the opinions of the visitors of two zoos, one in Italy and one in Germany, on drivers that may influence the ethical reputation of zoos. Preliminary results based on the answers of 274 respondents show that visitors' opinions on zoos acting with ethical responsibility are correlated with emotional appeal and familiarity with these institutions. The application of ZERS can help zoos identify weaknesses in their reputation and develop new strategies to improve people's attitudes towards them, bringing many benefits to the individual zoo and zoological institutions in general.

Introduction

More than 700 million people, one-tenth of the world population, representing a wide variety of demographic categories, visit zoos every year (Bruni et al., 2008; Moss et al., 2015; Stevens & McAlister, 2003; World Association of Zoos and Aquariums (WAZA), n.d.). With such vast and wide-ranging audiences, zoos can play an important role in educating children and adults on the importance of biodiversity and raising awareness of conservation challenges (Moss et al., 2015). Zoos are facilitated in their role by the fact that, while providing an entertainment experience, they create in visitors an emotional connection with animals and their stories (Mann-Lang et al., 2016; Myers et al., 2004). Moreover, the zoo experience itself provides visitors with implicit emotional connections with Nature as these institutions represent the first—and often the only—place where people can encounter many different species of wild animals (Bruni et al., 2008). These emotional connections are important because they have been seen to generate a

motivational stimulus that eases the learning of ethological and ecological contents, making visitors more receptive to conservation messages (Bromley, 2001; Bruni et al., 2008; Clayton et al., 2009; Dwyer et al., 2020; Fraser et al., 2007; Powell & Bullock, 2015).

Over the years, zoos have progressively assumed active and prominent positions in wildlife research and biodiversity conservation, supporting an integrated approach to species protection, like the One Plan Approach (Byers et al., 2013; IUCN Species Survival Commission (IUCN SSC). This conservation strategy—in which zoos play a relevant role—helps to bridge the gap between wild and captive population management, involving all conservationists (e.g., field biologists, wildlife managers, zookeepers, etc.) to develop a shared planning tool useful for species conservation (Byers et al., 2013; Minteer & Collins, 2013). However, to fulfill their mission, zoos must be trustworthy and credible in their role. For this reason, they need to have a good reputation among the public and other stakeholders.

The concept of the reputation of a zoo can be regarded as the application to zoological institutions of the well-known marketing concept of corporate reputation. According to Fombrun and Van Riel, corporate reputation is a collective representation of a firm's past actions and results that describes the firm's ability to deliver valued outcomes to multiple stakeholders (Fombrun & van Riel, 1997). Similarly, the reputation of a zoo can be defined as the collective representation of its past actions, commitment, and ability to fulfill its mission. It represents the general esteem in which the zoo is held internally by employees and externally by its stakeholders.

Reputation is considered an intangible but highly valuable asset. Indeed, studies have shown that corporate reputation has surpassed traditional palpable assets in determining the ability of a company to thrive because it attracts public support and more and better resources (Fombrun, 2006; Kaur & Singh, 2018). Likewise, also for zoological institutions, a positive reputation can produce several benefits. For instance, zoos with a positive reputation can attract more visitors, build loyalty, gain their trust and support for their conservation projects, be more effective in their pro-conservation messages, and have easier access to funds. As a result, a positive

reputation can fuel a positive "reinforcement loop" that facilitates the fulfillment of their institutional mission (Figure 1).

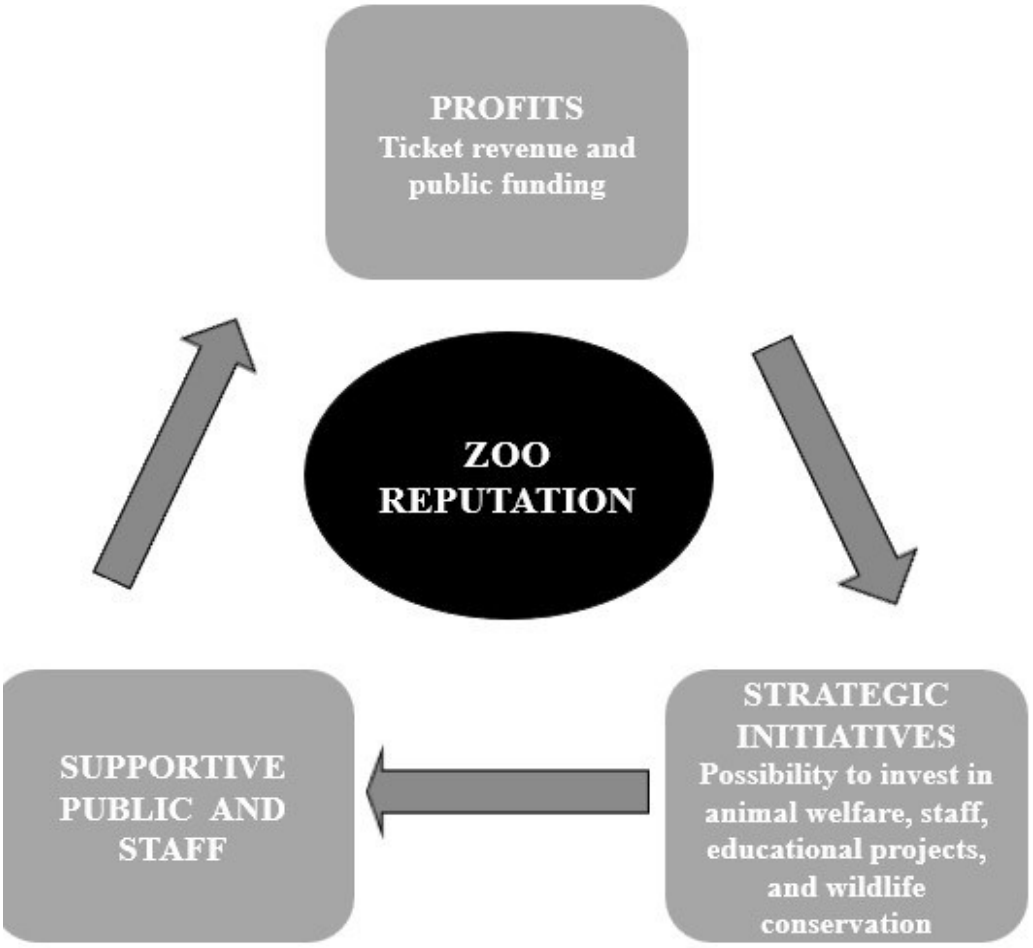

Figure 1. The reputational value cycle of zoos. A good zoo reputation can act as a positive reinforcement loop engine. It will ensure supportive public and staff, attract more visitors and revenue, and provide access to public funding. These will allow investment in strategic initiatives (animal welfare, staff, educational projects, and wildlife conservation), enabling the zoo to act according to its mission.

Furthermore, the benefits of a positive reputation reflect not only on the individual zoo but also on the whole zoo community. It may lead to a virtuous cycle in other zoos, encouraging them to operate at the highest standards and act ethically. Above all, the ethical aspects involved in the activities of zoos are becoming progressively crucial in contributing to a good reputation of these institutions as 'ethical arks' (Maple & Perdue, 2013a). These aspects can be listed as, for example, acting responsibly towards their mission, promoting individual animal welfare while enhancing the chance for the conservation of species, promoting transparency within the public in educational efforts, and selecting to adhere to conservation projects based on common ethical standards (Keulartz, 2015; Maple & Perdue, 2013b; Minteer & Collins, 2013; Minteer & Rojas, 2018; Norton et al., 1995; Stevens & McAlister, 2003). Zoological associations can benefit from analyzing and monitoring the reputation of their members and setting high ethical and reputational standards to which they must adhere.

Only zoos with a good reputation are considered credible in their actions as institutions for biodiversity protection and education by visitors, the general public, and the social networks in which they operate. Hence, there is an increasing need for zoological associations and individual zoos to be able to identify the crucial aspects that may influence their reputation. To our knowledge, currently, there are no existing tools able to evaluate the reputation—and specifically the ethical reputation—of zoos among visitors. Therefore, we designed an ad hoc survey, the Zoo Ethical Reputation Survey (ZERS). Here, we present its development and the results of its first trial in two zoos, one in Italy and one in Germany.

Method

The Conceptual Framework of ZERS

The first step in the design of ZERS consisted of a literature review on corporate reputation. The literature on the topic was retrieved from Scopus and Google scholar using the Boolean strings of the following combination of keywords ("corporate" or "zoo" or "zoos" "zoological garden" or "zoological gardens") AND "reputation". The retrieved articles were analyzed to identify the reputational key drivers, that is, the factors that drive corporate reputation by influencing and shaping it. Subsequently, the literature on each identified key driver was further investigated, and the concepts found were adapted to the context of zoological institutions.

There are many theoretical frameworks concerning possible drivers for reputation, with no consensus on their real action and effectiveness. The difficulty in identifying which drivers influence reputation unambiguously is partly due to the fact that a universal and operational definition of reputation is lacking because the concept needs to be defined each time for different contexts (Fombrun, 2012; Fombrun & van Riel, 1997; Kaur & Singh, 2018). This is particularly evident in zoos, which are very complex entities dealing with multiple stakeholders with very different and sometimes contradictory interests (e.g., individual animals, visitors, wildlife species, social communities, etc.). Consequently, many, often interconnected, factors can affect the reputation of zoos among the public.

For the development of ZERS, four types of drivers that may affect visitor opinions were considered: functional drivers, motivational drivers, relational drivers, and third-party influence drivers (Figure 2). Moreover, particular attention was paid to the ethical aspects concerning the activities of zoos. Analyzing and addressing the most pressing ethical issues concerning zoos is crucial not only to give deeper meaning to the maintenance of wildlife in these facilities but, above all, not to provide ammunition to those who oppose the very existence of zoos (Stevens & McAlister, 2003).

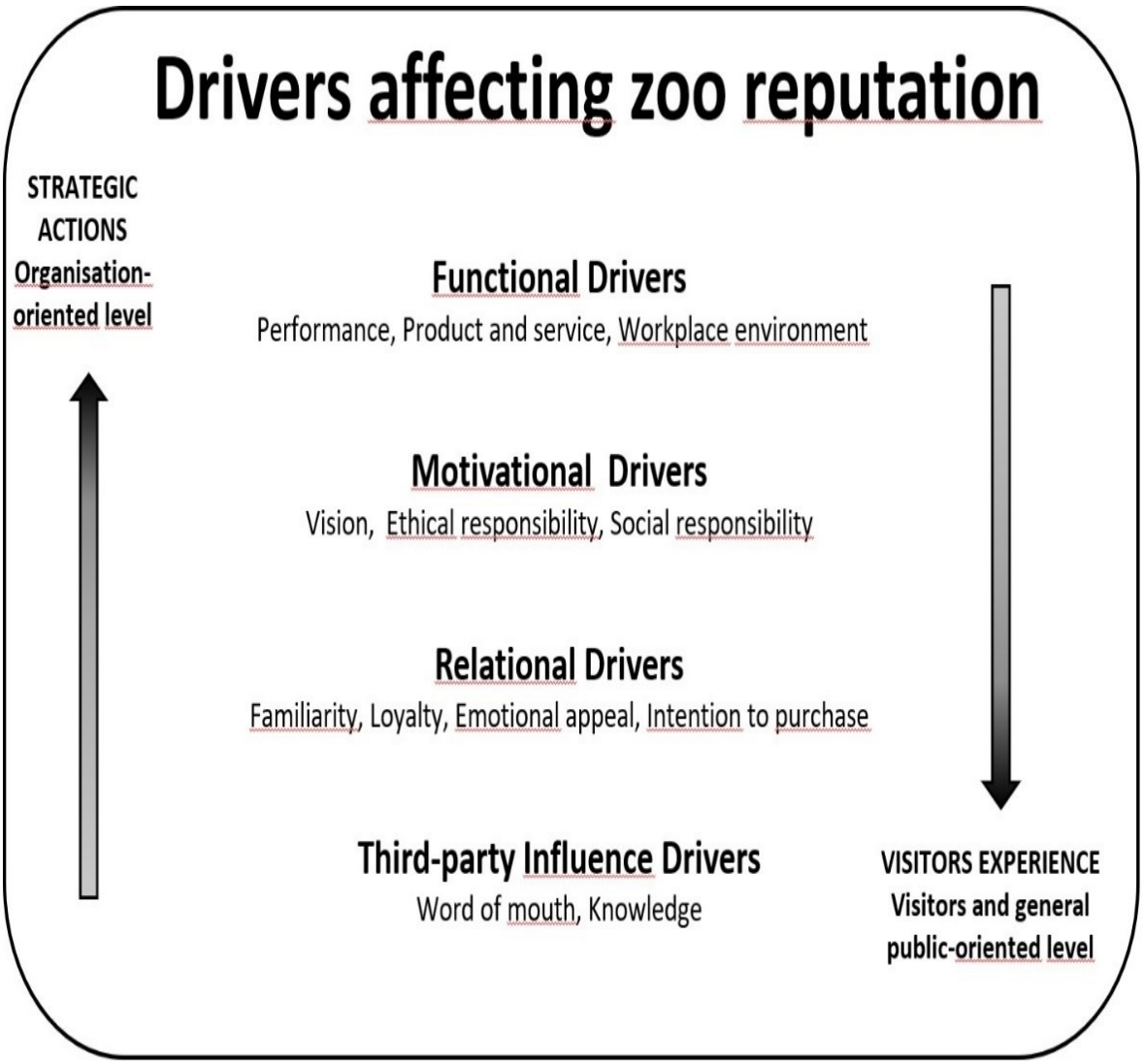

Figure 2. Drivers affecting zoo reputation analyzed in ZERS.

Functional Drivers

Functional drivers are related to the running of zoos and are the most widely researched in zoo management. They are affected by visitors' experiences of products, services, performance, and the working environment of the zoo, and they give the perceptions of the quality, innovation, value, and reliability of the institution's products and services (Fombrun & Foss, 2001). The performance represents the potential and ability of an organization to efficiently utilize the

available resources to achieve targets in line with the set plans, keeping in mind their relevance to the stakeholders (Peterson et al., 2003).

For a zoo, this means achieving the goals of its mission taking into consideration visitor satisfaction. The performance evaluation of a zoo is very important for investigating the quality of animal exhibits, husbandry and care of the animals, educational programs, and conservation projects. The analysis of the performance can help zoos maximize their education and conservation activities, encouraging them to work at higher standards and identify particular issues or concerns (Guadagnolo, 1985; Scott, 1993). In addition, setting performance benchmarks can also help improve individual institutions and the zoological industry as a whole (Bartos & Kelly, 1998). Moreover, the performance of a zoo is connected to the employees' working conditions and satisfaction. Specifically, good working conditions promote a connection between the employees, the zoo, and its mission. Subsequently, there will be less turnover, and the higher level of skills and know-how of employees will positively impact the performance of the zoo. Furthermore, the public will be more likely to believe that the institution and its workers are credible and dedicated to their mission (Alniacik et al., 2011).

Zoos are also places of entertainment, and customers who visit them expect to have a pleasant time there. Therefore, a positive experience of the performance, products, and services of the zoo during the visit significantly influences visitors' satisfaction, their intent to revisit, and their opinion about the reputation of the zoo (Sukwadi & Yang, 2014; Wu et al., 2017).

Motivational Drivers

Motivational drivers are related to the vision of the zoo and its social and ethical responsibility. Vision integrates the mission, the purpose of the organization, and values into a cohesive action-oriented plan (Van Wart, 1996). Especially, the mission of the zoo should be clearly expressed and declined in action-oriented language so that their accomplishment can also be evaluated by the general public (Patrick & Caplow, 2018). The adherence of the zoo to its stated vision and the achievement of its goals can significantly influence public opinion and, consequently, the

reputation of the zoo. Furthermore, zoos should cultivate a relationship with visitors to encourage them to identify with their mission to entice them to participate in their conservation efforts. However, the good reputation of a zoo is also established by the social role it can play and its ethical responsibility. In particular, its commitment to social and ethical responsibility is crucial. Zoo social responsibility is the ability to promote projects involving local communities and be an environmentally responsible organization. A corporation that acts according to socially responsible principles and practices is perceived as a good citizen in its dealings with the community, employees, and the environment, and its reputation will undoubtedly benefit from this (Fombrun, 2005). Similarly, also the ethical responsibility of a zoo significantly impacts its reputation. Acting according to ethical responsibility leads zoos to operate transparently, be open and accurate when disseminating information, and be committed to advancing superior animal welfare standards and practices (Maple & Perdue, 2013a; Minteer & Rojas, 2018).

Relational Drivers

The relational drivers that can influence the reputation of a zoo are related to the relationship with its visitors, such as its emotional appeal among the public and the familiarity and loyalty of its visitors, as well as visitors' repurchasing intentions. Zoos should create an emotional bond with their visitors so that communication of the pro-conservation messages can reach not only their minds but also their hearts (Barongi, R., Fisken, F. A ., Parker, Gusset, 2015). This emotional bond motivates visitors towards a personal commitment to Nature through donations to support projects carried out by zoos, as has been observed for other organizations (Paxton et al., 2020). More importantly, this affective component generates a place attachment. This loyalty to a particular zoo can be easily translated into a familiarity with zoological institutions in general, which increases esteem in these organizations and the likelihood of revisiting or visiting other zoos in the future and even recommending them to others (Ajayi & Tichaawa, 2021; Sinh & Anh, 2020; Tomas et al., 2002). Any zoo should succeed in creating this attachment in its visitors because this will facilitate the achievement of its mission. Indeed, research suggests that

repeat visitors are more likely to seek conservation efforts than those visiting zoos for the first time (Clayton et al., 2017; Godinez & Fernandez, 2019; Moss et al., 2017).

Drivers of Third-Party Influence

Third-party drivers that can influence the reputation of a zoo are related to the multi-way communication between the zoo and visitors, the general public, zoo networks, etc. Therefore, a zoo must know what kind of information is provided about it and how it is spread. Especially the dissemination of information through direct word of mouth among acquaintances significantly impacts reputation, as opinions conveyed in this way are often considered more trustworthy than those reported by other sources (Murray, 1991; Williams et al., 2012). Recently, this way of disseminating information has become even more relevant in shaping reputation because, through the Internet, electronic word of mouth (eWOM) can be spread globally, even among people who have never met each other, with a greater effect. Moreover, the more people publicly share that opinion, the bigger will be the number of people who agree with it. This is caused by a psychological phenomenon known as the "bandwagon effect"which generates a mechanism of social self-reinforcing in which the spreading of an opinion by the majority induces individuals to adopt that opinion as their own regardless of its veracity (Wang et al., 2015).

ZERS frame

As previously described, the review of the corporate reputation literature allowed us to select the categories of drivers that could be used in the analysis of the reputation of zoos. These drivers were utilized to define the ZERS outline (Figure 3), and, for each driver, the most critical issues that can influence the reputation of a zoo were highlighted and analyzed.

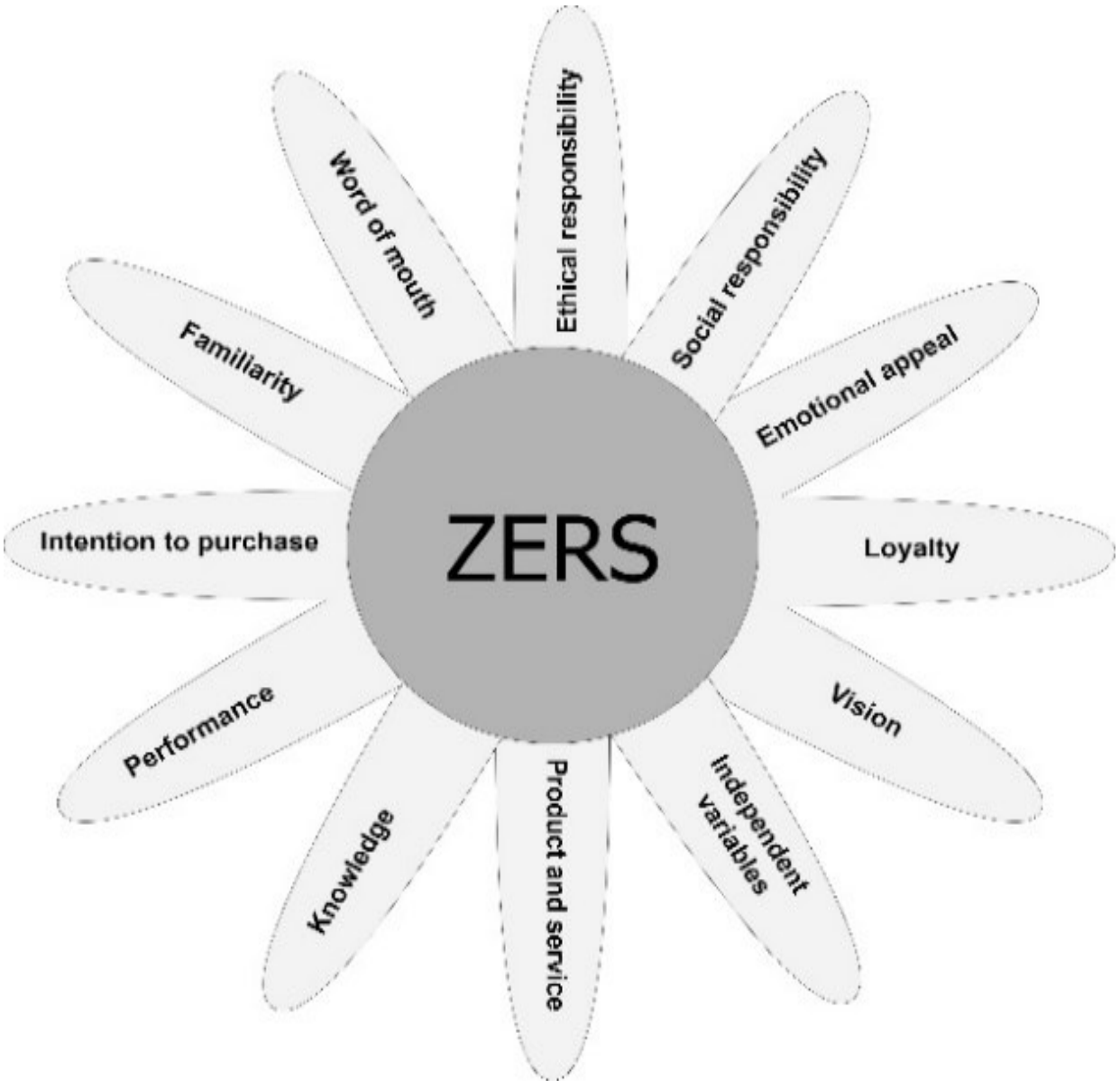

Figure 3. ZERS outline shows the drivers influencing the reputation of a zoo analyzed in the tool.

Consequently, we inserted 53 items in the ZERS survey to reflect these facets and were used to measure the opinion of visitors with the aim of implementing relevant strategies to address them. Furthermore, 9 additional questions were inserted to record their demographic characteristics. A challenging questionnaire in length for respondents but similar in length to questionnaires created to investigate the corporate reputation of other institutions (European Food Safety Authority (EFSA), 2020). We applied a psychometric methodology to formulate different kinds of items (i.e., closed-ended multiple-choice questions, rating scale questions, and Likert scale questions) depending on the type of information to be collected by the interviewees (Price, 2017). In the survey, the 5-point Likert scale items assessed the visitors' attitudes (options ranging from Strongly Disagree, Disagree, Neither Agree nor Disagree, and Strongly Agree). While we used a rating scale ranging from 1 (not at all likely) to 5 (extremely likely) to measure opinions such as the likelihood that visitors would recommend zoological institutions or visit a zoo in the future. In the questionnaire, the items were not subdivided or ordered according to the different categories shown in Figure 3 but according to the order considered easiest for respondents to answer. In any case, they were placed in such a way that respondents could not figure out to which reputational drivers they were referring to avoid response bias. Table 1 shows some of the questionnaire items for each specific facet.

4502 **Table 1.** ZERS questionnaire layout with items for each specific drive. The complete questionnaire is available in supplementary

4503 materials (S1).

4504

Drivers Category	Specific Driver	N.	Item
FUNCTIONAL DRIVERS	PERFORMANCE (PERF)	17	Zoos are committed to guaranteeing high standards of animal welfare
		18	Zoos educate their visitors about wildlife conservation
		19	Zoos do scientific research
		21	Zoos dedicate themselves to conservation projects in the wild
		31	Zoos are going to become a bigger reality in the future
	PRODUCT AND SERVICE (PR_SR)	12	Zoos enable a direct experience of wild animals
		20	The time spent in zoos is a good value for the money spent on the ticket
		27	Zoos' staff helped me in having a nice day at the zoo
	WORKPLACE (WORKP)	24	Zoos' staff is passionate about their job
		25	Zoos are well managed
		26	Zoos are good companies to work for
MOTIVATIONAL DRIVERS	VISION (VISION)	34	Zoos make unclear and undefined promises
		35	Zoos have excellent management
		36	Zoos clearly explain their goals and their mission
		34	Zoos make unclear and undefined promises
		35	Zoos have excellent management
	ETHICAL RESPONSIBILITY (ETR)	23	Zoos act in a transparent and ethical way
		33	Zoos are open and transparent about the way they operate
		37	Zoos are accurate when disseminating information
		38	Zoos do what they say they are going to do
		39	Zoos are dishonest and false in their communications
	SOCIAL RESPONSIBILITY (SOCRES)	29	Zoos are environmentally responsible organizations
		30	Zoos support good causes
		32	Zoos handle their animals in a responsible way
RELATIONAL DRIVERS	FAMILIARITY (FAM)	1–5	How many times have you visited the following facilities in the last 12 months? Zoos Aquariums Natural parks and reserves Safari parks Other facilities that house wild animals
		6	Rate your degree of familiarity with zoos
	EMOTIONAL APPEAL (EMA)	8	I trust zoos
		9	I have negative feelings toward zoos
		10	Zoos have a good reputation
		11	I admire and respect zoos
		13–16	How frequently do you feel each of these emotions when thinking about animal extinctions? Worried Alarmed Unconcerned Hopeful
		52	I will leave feedback about how the zoo can be improved
		53	If a zoo has to face a problem, I trust it will make the right choice
	LOYALTY (LOY)	7	Do you have a season ticket or a membership pass for a zoo?
	INTENTION TO PURCHASE (ITP)	49	What's the likelihood that you will visit zoos in the future?
THIRD-PARTY INFLUENTIAL DRIVERS	KNOWLEDGE (KNOW)	22	Are animals in zoos taken from the wild?
	POSITIVE WORD OF MOUTH (PWM)	50	I will suggest to a friend to go to zoos
		51	I will say positive things about zoos

4505

The Administration of ZERS

The first trial of ZERS was in a two-site cross-sectional observational study, a method used to compare the opinions of two different groups of zoo visitors at one point in time (X. Wang & Cheng, 2020). Specifically, ZERS was administered to visitors in two European zoos: the Zoological Gardens of Pistoia in Italy and the Opel Zoo in Germany. The researchers administered the survey to visitors following a random sampling procedure and fairly sampled visitors that passed an imaginary line in front of them (Acharya et al., 2013; X. Wang & Cheng, 2020).

All the participants were informed of the purpose of the research, and verbal consent was requested when they were invited to take part in the study. Permission from responsible adults was sought before potential respondents of minor age were approached. No anticipated risks to the participants were identified as they were invited to take part voluntarily and anonymously in the study at the entrance of the zoo. Furthermore, to ensure anonymity, no personal data that could link the questionnaire to the respondent's identity in any possible way were collected. The administration of the questionnaire took place in both zoological institutions, for approximately seven hours per day, on 2nd and 3rd June 2018, from 10 a.m. until closing time.

Methods and Reliability Analysis

The research hypothesis had a twofold focus: to analyze how visitors in the two different zoos perceive the reputation and ethical aspects of the activities of the zoos and to investigate which drivers influence them.

Propaedeutically to the data analysis, a study of ZERS questionnaire reliability was performed to identify which dimensions to retain. R. and Jamovi software were used for all analyses and plots (R Core Team, n.d.; The Jamovi Project, n.d.). For this purpose, Cronbach's coefficient α was used to calculate the internal consistency coefficients of the scales. This coefficient represents how closely related a set of items are as a group, that is, how stable measurement is, as it is a requirement for validity.

As shown in Table 2, the 95% confidence intervals of Cronbach's α for all the drivers/dimensions include a parameter of around 0.70 (except in the case of the Loyalty driver). Given the early stage of this construct validation research, such reliability value was considered satisfactory, although modest for Nunnally and Bernstein standards (Nunnally & Bernstein, 1994).

Table 2. Reliability Scale of ZERS drivers. Crombach's α CI values ranging from 0.70 to 0.85 are considered acceptable**.**

		95.0% Confidence Interval	
	Cronbach's α	**Lower**	**Upper**
Ethical responsibility	0.848	0.812	0.870
Familiarity	0.694	0.616	0.734
Loyalty	0.148	0.080	0.391
Workplace	0.703	0.634	0.757
Performance	0.754	0.705	0.797
Social responsibility	0.754	0.702	0.802
Emotional appeal	0.767	0.712	0.805
Extinction awareness	0.696	0.643	0.763
Vision	0.675	0.60	0.736

As previously stated, reliability is a necessary condition for validity, but it does not imply it. Although the numerosity of respondents did not provide the opportunity for a more advanced statistical analysis of the ZERS validity, the correlation among key drivers was used to test our hypothetical pattern. Based on the theoretical development of the ZERS tool, if the drivers were valid in the measurement, we expected a stronger relationship between all other variables, as theoretically hypothesized. In fact, the correlation matrix (Table 3) provided indications of a

statistically significant moderate positive correlation between ethical responsibility (ETR) and emotional appeal (EMA), r(263) = 0.581, $p < 0.01$, indicating how the perception of zoo mission can also activate emotional arousal in the visitors (and vice versa). Similarly, the small positive correlation between familiarity zoo-related (FAM_ZOO), r(239) = 0.133, $p < 0.05$, and familiarity with other settings such as parks and aquariums (FAM_NO-ZOO), r(235) = 0.335, $p < 0.01$ was expected because it intercepts the profile of people who like visiting natural attractions. All the other correlations between the selected key drivers are smaller but statistically significant, confirming that they represent different, but related dimensions of the zoo reputation construct.

Table 3. Correlation matrix. The correlation matrix was used to test the hypothetical relationship pattern among selected key drivers. The results provide indications of statistically significant correlation between Ethical responsibility with all other variables, moderate positive correlation with Emotional appeal, r (263) = 0.581, $p < 0.01$, and small correlation with Familiarity zoo-related r (239) = 0.133, $p < 0.05$ and Familiarity not zoo-related r (235) = 0.148, $p < 0.05$. * Pearson Correlation $p < 0.05$ level (2-tailed). ** Pearson Correlation $p < 0.01$ level (2-tailed).

	ETR	EMA	FAM_ZOO	FAM_NOZOO
ETR	1			
EMA	0.581 **	1		
FAM_ZOO	0.133 *	0.164 *	1	
FAM_NOZOO	0.148 *	0.053	0.335 **	1

This evidence was considered to support the data analysis related to the questionnaire dimensions, except for the Loyalty driver, which was considered biased and was not taken into further consideration.

Results

Three hundred thirty-three respondents filled out the questionnaire. After the data screening (checking for missing data, uncompleted or unengaged responses, etc.), the final dataset analyzed comprised 274 data points: 89 (32.8%) in Germany and 189 (67.2%) in Italy. This step of data analysis can also be considered a preliminary phase, as it regards the comparison of the two populations to highlight relevant differences. This comparison can provide additional insight into the discussion of the results related to the ZERS drivers.

To investigate the socio-demographic characteristics of the visitors surveyed, respondents of the two different zoos were compared on the main variables using the chi-square test of independence. The two groups demonstrated statistically significant differences in gender (χ^2 = 24.45, $p < 0.001$), with 52.2% male respondents in the Italian zoo and 31.7% in the German zoo. This difference in gender proportions in the two populations highlighted by the Chi square statistics is relevant because literature reports gender differences in customer expectations and perceptions of corporate social responsibility in other contexts (Calabrese et al., 2016). Moreover, visitors of the Italian zoo had a statistically significantly higher age (rrb = −0.63, $p <$ 0.01), with a median age of 35–54 years, while the median age of visitors of the German zoo was 26–34 years. Rank-biserial correlation value between one nominal variable (nationality) and one continuous one (age) is important because age can affect some reputation drivers, as shown by our results, a little further. Therefore this may explain the higher mean scores of the items. Moreover, the education level of the visitors to the Italian zoo was significantly higher (rrb = −0.21, $p < 0.01$), with 82.3% of Italian visitors having a secondary school diploma or a higher education compared to 66.9% of the visitors of German zoo, but a lower income (rrb = −0.385, $p < 0.01$), with Italians having income level median of 14,000−29,999 € and Germans of 30,000−40,000 €. Education and income levels did not appear relevant for reputation drivers in the following analysis. Therefore, these differences could be negligible.

A descriptive analysis of the responses to single items was also conducted to better comprehend the participants' perception, and to test the usefulness of ZERS tool in this trial. Additionally,

supplementary evaluations on the responses in the two zoos were conducted on some ad-hoc selected items using the Mann–Whitney U test, because the variables were considered as ordinal in nature. For all these items, the mean value of the Italian population was higher than the German one; in fact, the W scores are positive, but only a few of these differences are statistically significant (Table 4). For example, question 21, reflecting performance driver ($p < 0.001$), shows how Italian respondents perceive that "Zoos dedicate themselves to conservation projects in the wild" more than the German group. This information could be used, for example, as leverage in media campaigns, etc.

More results are described in Table 2, and further descriptive analysis are reported in the Figures 4, 5,6,7.

Figure 4. Descriptive analysis ofthe responsenses to the items that were assessing the public opinion on the drivers linked to the performance of the zoos.

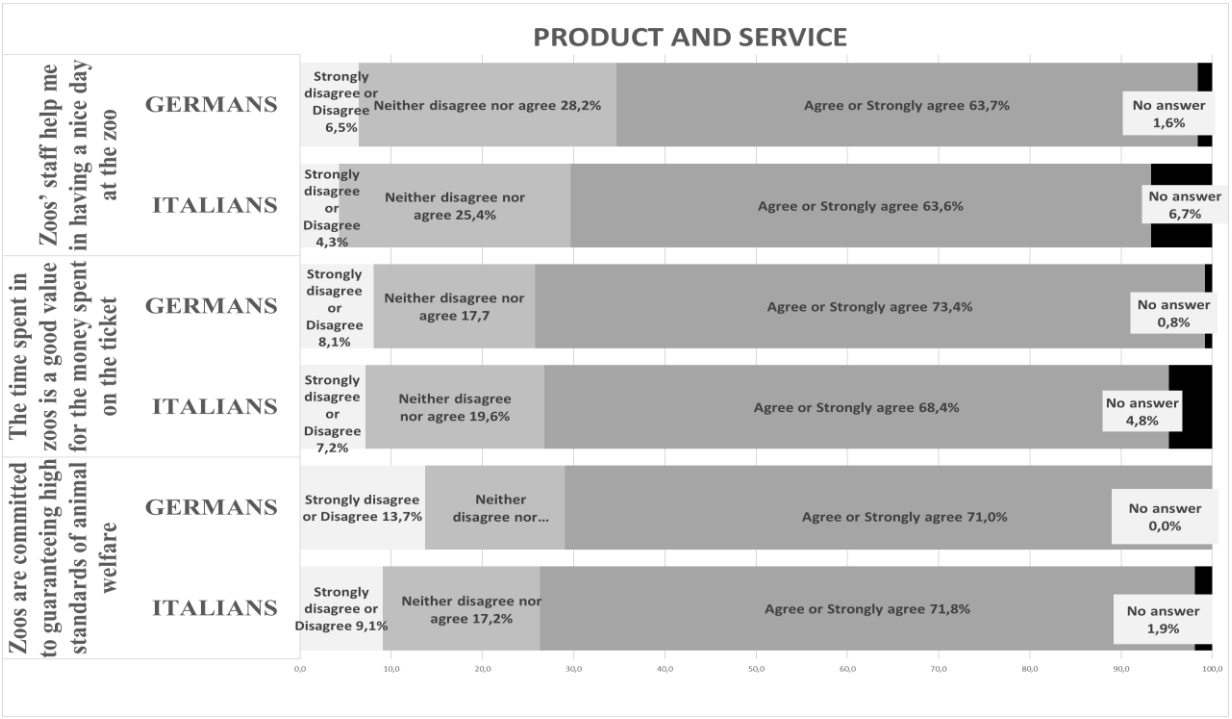

Figure 5. Descriptive analysis of the responsenses to the items that were assessing the public opinion on the drivers linked to product and service offered by zoos.

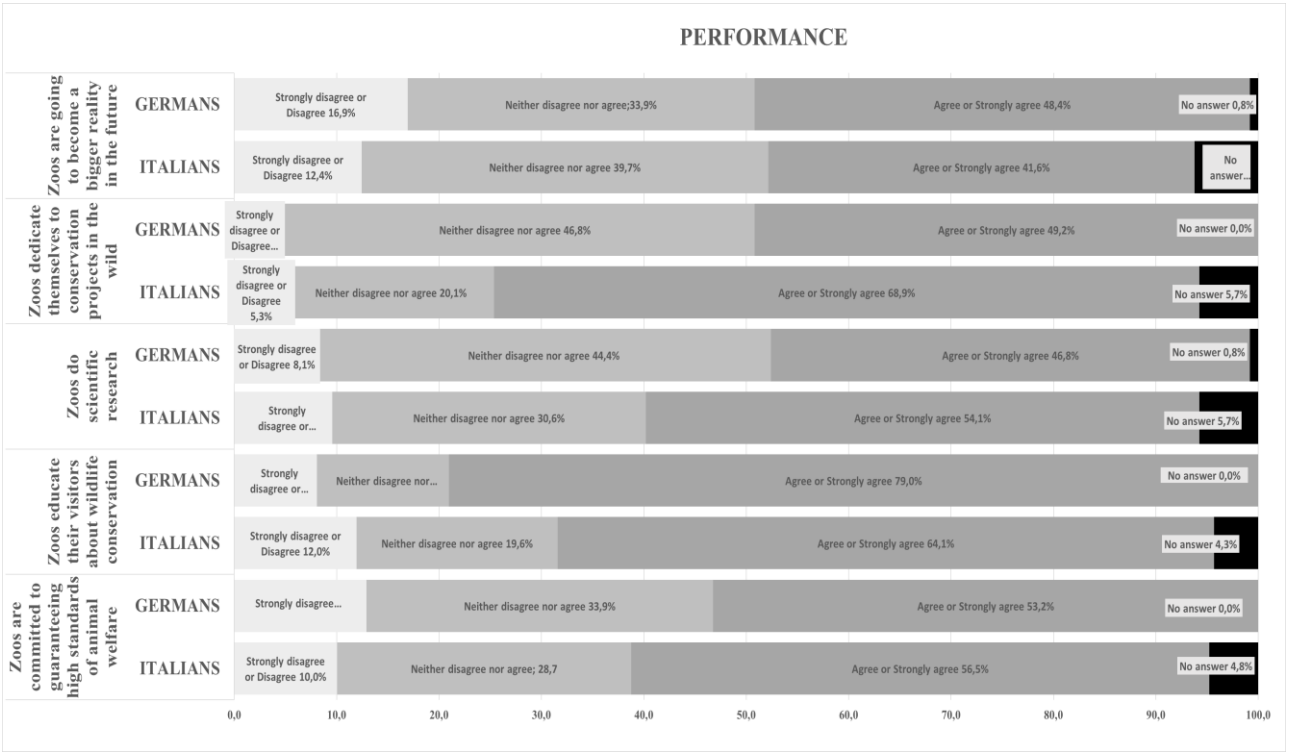

Figure 6. Descriptive analysis of the responsenses to the items that were assessing the public opinion on the drivers linked workplace.

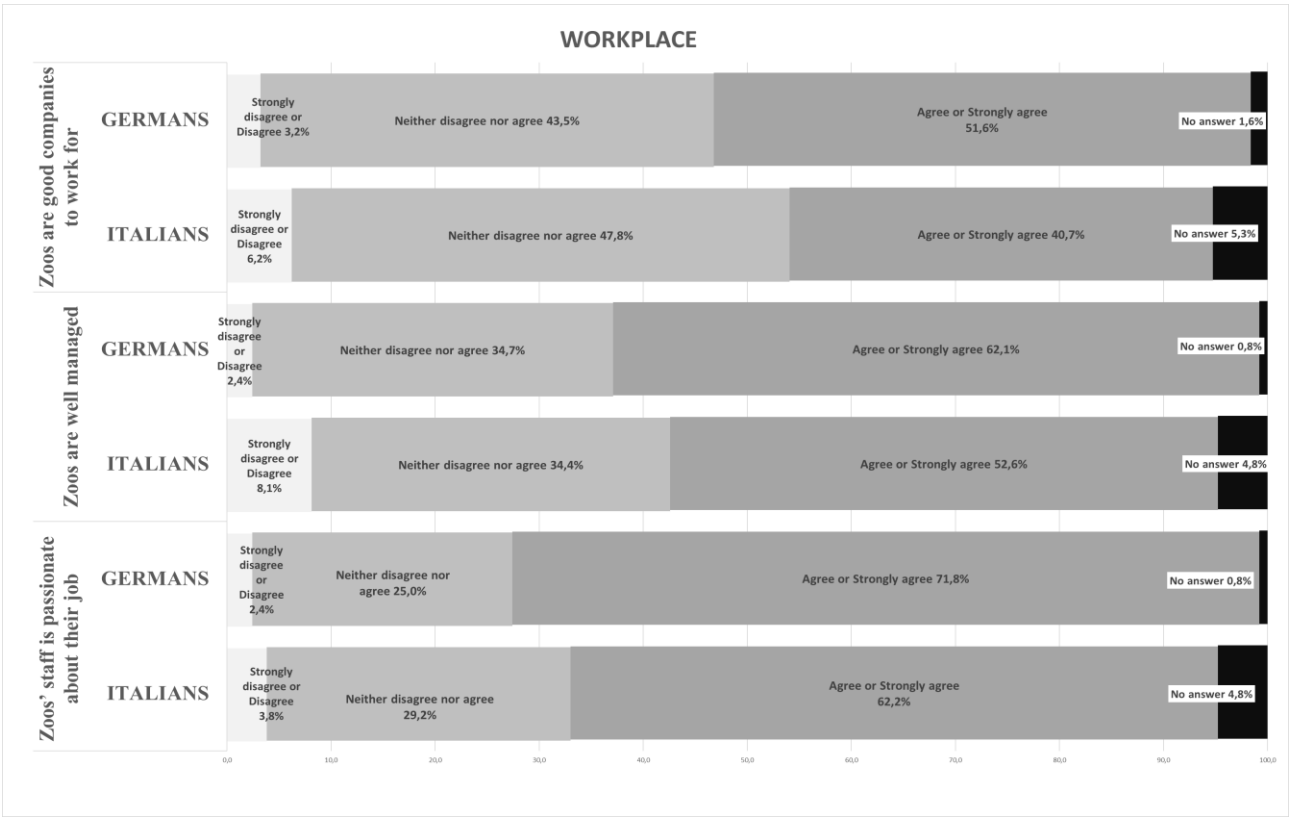

Figure 7. Descriptive analysis of the responsenses to the items that were assessing the public opinion on the drivers linked the vision of the zoos.

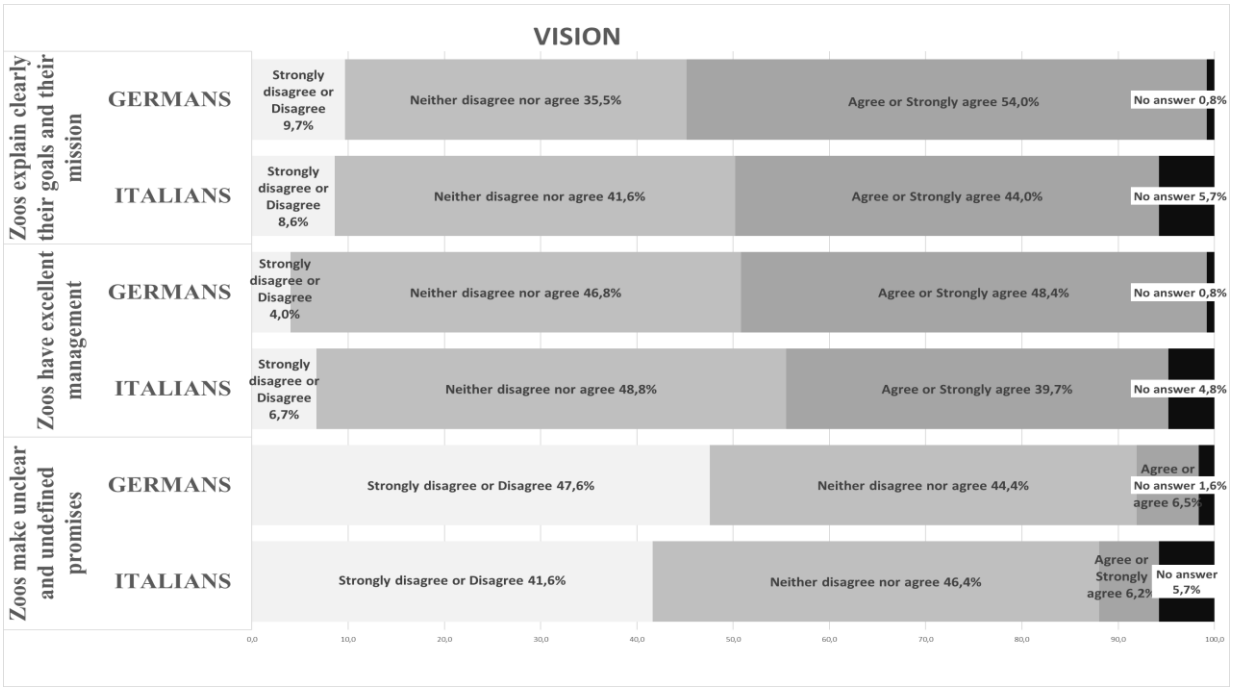

Table 4. Mann–Whitney test parameters for selected questionnaire items. Example of the item coding system: QXX_ETR = Q (question) × X (item order in the questionnaire), _ETR (item-related driver). For the Mann–Whitney test, the location parameter is given by the Hodges–Lehmann estimate. Levene's test is significant ($p < 0.05$), suggesting a violation of the equal variance assumption (it may determine a bias in the interpretation).

	W	P	Hodges–Lehmann Estimate	Rank-Biserial Correlation
Q24_WORKP	9444.50	0.04	3.15e-05	0.14
Q23_ETR	10,399.50	9.71e-05	5.91e-05	0.26
Q6_FAM	4798.00	2.06e-05	−1.00	−0.31
Q10_EMA	9168.00	0.09	4.74e-05	0.12
Q11_EMA	9526.50	0.03	4.22e-05	0.16
Q51_PWM	11,299.00	9.96e-08	1.00	0.38
Q18_PERF	8782.50	0.37	1.34e-05	0.06
Q19_PER	8834.00	0.29	1.65e-05	0.07
Q21_PERF	11,817.50	3.36e-10	1.00	0.44

Next, the different effects between nationalities on the relevant drivers (continuous variables) regarding visitors' opinions on familiarity (FAM), ethical responsibility (ETR), and emotional appeal (EMA) were checked with gender as a grouping variable, using the Independent Samples T-Test (Table 5). Regarding visitors' opinions, the differences between nationality on the relevant drivers (continuous variables) on familiarity (FAM), ethical responsibility (ETR), and emotional appeal (EMA) were checked with gender as a grouping variable, using the Independent Samples T-Test. The objective, in this case, was to verify a possible effect of gender within the nationality. The results presented in Table 5 confirm for all the drivers (except for

familiarity) a higher statistically significant perception for male visitors versus female ones (positive mean difference and p-value < 0.05). Zoos could evaluate this evidence to reflect on the reasons why there is this difference and how to intervene to raise the perception of female visitors.

Table 5. Independent Samples T-Test on the differences between nationality on the relevant constructs checked with gender as a grouping variable. For the Student t-test, the effect size is given by Cohen's d. For the Student t-test, the location parameter is given by the mean difference.

						95% CI for Mean Difference		
	t	df	*p*	Mean Difference	SE Difference	Lower	Upper	Cohen's d
FAM	2.090	215	0.038	−0.950	0.454	1.845	0.054	−0.299
ETR	4.928	265	<0.001	2.112	0.429	1.268	2.956	0.640
EMA	4.005	268	<0.001	1.117	0.279	0.568	1.666	0.517
PWM	5.65	271	<0.001	2.04	0.36	1.33	2.76	0.73
WORK	2.79	271	<0.001	0.65	0.23	0.19	1.10	0.36
PERF	5.21	268	<0.001	1.99	0.39	1.22	2.75	0.66

Further analysis with two-way ANOVA highlighted differences in familiarity (FAM) considering the nationality and gender variables at the same time. The statistic coefficients showed that while the principal effects of the independent variables (the "gender" and "nationality" rows) are not statistically significant, their interaction (the "GENDER ✻ NATIONALITY" row) is determining an effect (p = 0.027) on the dependent variable "Familiarity" (Table 6 and Figure 8). This result explains the opposite trend presented in Table

5 because the plot in Figure 8 shows a statistically significant difference in familiarity mean scores between German female visitors and Italian ones. This test value may be due to the unbalanced gender distribution in the German sample. Still, it may be worth investigating in the future because this opposite trend can be determined by other intervening variables (like a ticket price policy favourable to female visitors that increase their familiarity with these structures).

Table 6. Differences between nationality and gender on familiarity with visitors. The analysis was conducted with ANOVA Type III Sum of Squares.

Cases	Sum of Squares	df	Mean Square	F	p
GENDER	16.317	1	16.317	1.654	0.200
NATIONALITY	2.304	1	2.304	0.234	0.629
GENDER ✻ NATIONALITY	48.801	1	48.801	4.946	0.027
RESIDUALS	2091.737	212	9.867		

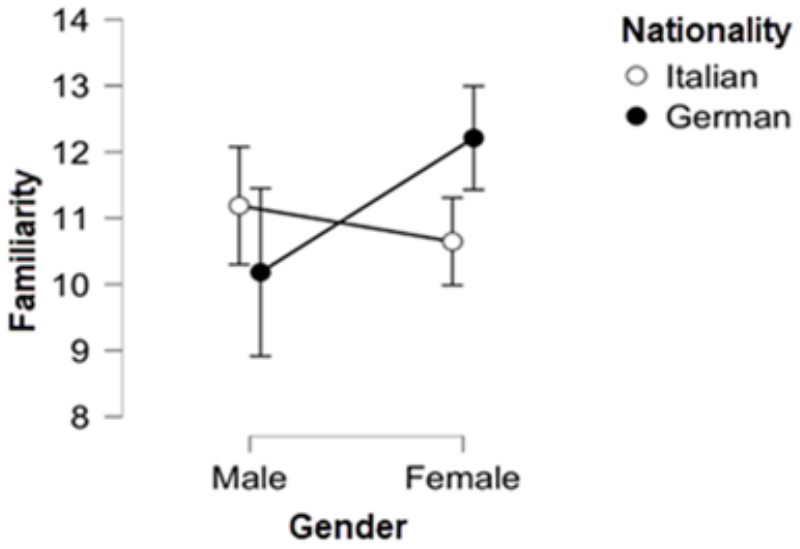

Figure 8. Descriptive plots of the statistically significant interaction GENDER ✻ NATIONALITY on familiarity. Axes: Y = Familiarity scores; X = gender.

Complementarily, Post Hoc Tests were conducted to evaluate the differences between the combination of gender and nationality of the respondents in the two zoos to complete the model description, as reported in Table 7. These results are more interesting when considering that the independent T-test on familiarity examining only nationality shows a higher mean for the German sample $t(215) = -2.090$, $p = 0.038$.

Table 7. Post Hoc Comparisons of gender respondents in the zoos. The *p*-value was adjusted for comparing a family of four using Tukey's correction**.**

		Mean Difference	SE	t	p_{tukey}
Male, Italian	Female, Italian	0.542	0.528	1.026	0.734
	Male, German	1.006	1.025	0.981	0.760
	Female, German	−1.022	0.560	−1.826	0.264
Female, Italian	Male, German	0.464	1.011	0.459	0.968
	Female, German	−1.564	0.533	−2.935	0.019
Male, German	Female, German	−2.028	1.028	−1.973	0.201

Finally, data of all respondents were analyzed as a whole, and two multiple regressions were run to predict differences in emotional appeal (EMA) and ethical responsibility (ETR) from gender, age, and education level (EDL), pet ownership (PETOWN), urbanization (URBANIZ), income level (INCOME), and zoo familiarity (FAM-ZOO). Both multiple regression model statistically significantly predicted the dependent variables (EMA: $F(7, 218) = 2.267$, $p = 0.03$, adj. R2 = 0.038; ETR: $F(7, 215) = 2.842$, $p = 0.007$, adj. $R^2 = 0.056$) with small effect sizes according to Cohen's classification (Cohen, 1988). In both models, age and zoo familiarity were found to be significant predictors ($p < 0.05$), and this consistency may indicate these are two variables

affecting the reputation construct as a whole. Regression coefficients and standard errors showed how an increase in zoo familiarity and age determines a rise in emotional appeal and ethical responsibility, as presented in Tables 8 and 9. The positive sign of the β coefficients indicates that older people with a better familiarity with the zoo also perceive more emotional appeal toward it and its ethical responsibility and vice versa.

Table 8. Results of the multiple regressions applied to predict differences in emotional appeal (EMA) from gender, age, and education level (EDL), pet ownership (PETOWN), urbanization (URBANIZ), income level (INCOME), and zoo familiarity (FAM-ZOO). In the table, the p-values < 0.05 indicate the corresponding variable is a statistically significant predictor of the outcome variable.

Emotional Appeal	**B**	**SEB**	***β***	**t**	**Sig.**
Gender	−0.115	0.313	−0.025	−0.369	0.713
AGE	0.464	0.163	0.204	2.854	0.005
EDL	−0.269	0.246	−0.076	−1.092	0.276
PETOWN	−0.014	0.299	−0.003	−0.047	0.963
URBANIZ	−0.152	0.167	−0.061	−0.910	0.364
INCOME	−0.018	0.087	−0.014	−0.207	0.836
FAM_ZOO	0.229	0.095	0.161	2.417	0.016

Table 9. Caption. Results of the multiple regressions applied to predict differences in ethical responsibility (ETR) from gender, age, and education level (EDL), pet ownership (PETOWN), urbanization (URBANIZ), income level (INCOME), and zoo familiarity (FAM-ZOO). In the table, the p-values < 0.05 indicate the corresponding variable is a statistically significant predictor of the outcome variable.

Ethical Responsibility	B	SEB	β	t	Sig.
Intercept	20.087	1.598		12.568	0.000
Gender	−0.287	0.491	−0.040	−0.585	0.559
AGE	0.852	0.257	0.237	3.310	0.001
EDL	−0.350	0.383	−0.064	−0.914	0.362
PETOWN	−0.222	0.466	−0.032	−0.476	0.634
URBANIZ	−0.328	0.268	−0.082	−1.223	0.223
INCOME	−0.270	0.137	−0.133	−1.975	0.050
FAM_ZOO	0.294	0.149	0.132	1.980	0.049

4. Discussion

The results of the preliminary analysis regarding the validity and reliability of the ZERS revealed a positive evaluation of both internal consistency and construct validity. This analysis confirmed the quality of the tool regarding eight scales/drivers and suggested a complete revision of the Loyalty scale, which presented an inadequate Cronbach's α value and, consequently, a low level of construct validity. Additional technical issues are reported in the limitation section. However, further testing is required to validate the instrument, collecting more numerous samples to implement more advanced psychometric methods and, ultimately, developing a quotient that can quantitatively measure the reputation of zoos among the public, as has been done for other corporations.

Furthermore, the responses to the questionnaires were analyzed for preliminary socio-demographic information of the respondents in the two countries presented some interesting differences. The results showed that the Italian respondents were mainly men and had statistically higher age, education level, and income. This is probably due to the fact that families with children often visit zoos, and Italians tend to have children later in life when they have completed their studies (Cook & Furstenberg, 2002). In other studies, it was observed that educational background and income level influence the extent to which people visit zoos (Davey, 2007; Whitworth, 2012). In fact, even if humans seem to be characterized by an innate "biophilic instinct" (Wilson, 1984), research shows that a higher level of education is correlated with greater interest and affection for Nature (Kellert, 1996). The presented results do not support this claim regarding the zoo reputation construct, although familiarity appears to influence its key drivers like Emotional Appeal and Ethical Responsibility. Nevertheless, apart from age, no other socio-demographic variable appears to influence key drivers. One explanation is that reputation of a zoo is a multi-facet construct that requires a long time to be acquired. Zoos and other stakeholders can use this information to calibrate their communication, e.g., it would be inefficient to focus on children to improve this construct.

Moreover, the differences in perceptions of the key drivers of the reputation of the zoo between male visitors and female ones were consistent across all the dimensions investigated and mirrored in the two national samples. Zoos could use these results to reflect on the reasons for this difference and how to intervene to increase the positive perception of female visitors on these key drivers.

In addition, the results suggest that Germans are more likely to have higher familiarity with zoological institutions. This is consistent with the fact that in countries like Germany, zoos are often public institutions, perceived as part of the social fabric, and frequented by all social classes. Not surprisingly, German zoos are the most visited in Europe (Davey, 2007).

Additionally, results showed a direct correlation between zoo familiarity and visitors' age with emotional appeal and ethical responsibility. From the theoretical point of view, it is an important

result for future studies on the topic because it rules out independent variables to be included in the next analytical model and differentiates for the specific research area. These findings suggest that familiarity with zoos, especially when cultivated over the years, as it may happen in older visitors, creates an emotional bond with these institutions that increases confidence that zoos act with ethical responsibility, thus improving their reputation.

The fact that emotional appeal showed a correlation with zoo familiarity is also particularly relevant. Although emotions are short-lived and context-specific, several studies claimed that they influence customers in creating their opinion on the reputation of a corporation (Andrade & Ariely, 2009; Groenland, 2002). Moreover, the results of the ZERS trial showed a positive—even if moderate—correlation between ethical responsibility and emotional appeal. Consequently, zoo managers should give special consideration to the fact that positive emotions experienced during a visit can influence the visitor's opinion about the reputation of that zoo as an ethical institution. To this end, zoo managers should pay special attention to explicit wildlife conservation efforts carried out by the zoo and promote emotionally engaging educational activities for visitors.

Furthermore, the analysis of the results of the individual ZERS items (see attachment 2) appeared promising, showing how zoos and their stakeholders can identify specific criticalities. For example, regarding the driver of zoo performance to question No. 18, "Do zoos educate their visitors about wildlife conservation?", several respondents answered that they strongly disagreed and disagreed or had no definite opinion on the statement (in Italy, 11% of respondents strongly disagreed or disagreed and 21% neither agree nor disagree, while in Germany 17% strongly disagreed or disagreed and 16% neither agreed nor disagreed). Similarly, regarding question No. 19, "Zoos do scientific research?", 31% of Italians and 43% of Germans showed that they had no definite opinion. On the other hand, visitors' opinions in the two zoos regarding question No. 21, "Do zoos engage in nature conservation projects," differed, with the majority of Italians (75%) agreeing or very much agreeing compared to Germans (34%) and, interestingly, with more than half of Germans (54%) having no definite opinion.

Analysis of responses to individual ZERS items can enable the zoo to highlight areas it can work on to improve its reputation. If the zoo has received a negative response on a specific item, it can use the result to assess whether this is due to an actual deficiency in that aspect or if, despite its correct actions, there is still a misperception by the public. For example, the above-mentioned responses highlight weaknesses in communication since most zoos spend money, make significant efforts, and employ staff dedicated to scientific research and conservation projects. Still, several visitors seem not to be aware of it. This is also confirmed by the answers to question No. 22, which suggest that many visitors still think that zoo animals are taken from the wild: "Always" for 1% of Italians and 0% of Germans; "Very often" for 8% of Italians and 12% Germans; "Sometimes" for 17% of Italians and 29% of Germans; and with 26.8% of Italians and 19. 40% of Germans "not knowing". Moreover, regarding the opinion on whether zoos are committed to maintaining animals to high welfare standards (item No. 17), 11% of respondents in Italy and 6% in Germany disagree or strongly disagree, and, remarkably, 29% of respondents in both countries do not have a definite opinion. All these features can significantly influence the reputation of zoos and the credibility of these institutions as agents of biodiversity protection, and when visitors were asked if they had negative feelings toward zoos, 50% of Italians and 38% of Germans agreed or very much agreed.

Additionally, when analyzing the answers concerning the fact that zoos act transparently and ethically, in question No. 23, a difference between the opinions of the respondents in the two countries (41% of the Italians and 22% of the Germans agree or very much agree) was highlighted. Again, 51% of Italians and 57% of Germans did not express a definite opinion. These results are particularly relevant because they show that, in the two zoos, a high percentage of visitors still need to form an opinion, and zoos should implement their actions on them. Notably, when asked directly whether they thought zoos had a good reputation, 42% of Italians and 41% of Germans respondents did not express a definite opinion, and only 37% of Italians and 30% of Germans agreed or strongly agreed. These responses show how significant it is for zoos to work on their reputation and how much work on this aspect needs to be done.

As shown above, the use of ZERS can provide zoos with several types of important information that may be relevant to finding strategies to improve the relationship between zoos and their stakeholders. Stakeholder management is an essential component of any business strategy in general, but it has only recently begun to be applied to zoological institutions. ZERS can be used not only to record and assess stakeholder opinions toward zoos but also to enable a more comprehensive understanding of the underlying reputational factors that elicit emotional attachment to zoological institutions. In addition, through analysis of simple descriptive statistics of individual items, the tool can be used to be focused on identifying specific critical issues that negatively influence visitor opinions. However, further applications are needed to better assess how much the type of visitors to different zoos, countries and cultural contexts influence the response recorded.

However, the outcomes of the first trial of ZERS questionnaire in two different European zoos showed that the tool helps investigate visitors' opinions on the drivers that can influence the reputation of zoos, and the information collected will be useful to refine the measurement tool.

Strengths and Limitations of the Tool and Future Developments

Reliability and validity analysis of the first trial of ZERS showed coherent and consistent evidence of its usefulness to assess the opinion of zoo visitors on the critical drivers that can determine the reputation of zoos on specific aspects of their activities and their ethical reputation. However, there are some study limitations to take into consideration.

Firstly, regarding the sample. Although the number of respondents was adequate for the study, considering that participants were not randomly selected and the questionnaire was administered only in one zoo in Italy and one in Germany, the results cannot be representative of the opinions of the entire reference population. However, this first trial of ZERS was useful in highlighting some critical issues, such as the length of the questionnaire. This has led to a revision, which is still in progress, to reduce the number of items and reword those difficult for respondents to

understand. After the revision, a wider sample will be necessary to correctly test the structure of the constructs (drivers or latent variables) included in the questionnaire. Moreover, to further improve this measurement tool, validating the questionnaire on zoo visitors in different countries will be crucial.

Finally, it should be considered that ZERS was designed to evaluate the opinion of only one of the stakeholders of a zoo—its visitors— but in reality, the reputation is a multi-dimensional construct that reflects the unique dimensions on which individual stakeholders base their judgments of an institution (Fombrun et al., 2000). Therefore, for a more comprehensive analysis —which would allow a zoo to better assess all the critical aspects that affect its reputation— it could be useful, in the future, to improve the tool in a way that may include the opinions also of other stakeholders (e.g., zoo worker zoological operators, environmentalists, local authorities, etc.).

Among the stakeholders, those who must be given special consideration are children. Indeed, children are perhaps the most important users of zoos, to whom the majority of the educational activities that zoos offer are dedicated. It would be very interesting for zoos to analyze children's opinions about their reputations. However, for this purpose, it will be necessary to design a suitable version of ZERS questionnaire. Specifically, the ZERS items will have to be adapted in number and wording to make them understandable to a younger audience.

Additionally, it will also be helpful to administer the ZERS questionnaire to assess the opinion of the non-visitor population, considering that almost no research exists to date comparing visitors versus non-visitors on many zoo-related topic. This would be of particular interest because it would help to explain if the ethical reputation of zoos can influence the propensity to visit zoos. Therefore, analyzing this population's opinion could help zoos find strategies to expand their visitor base.

In the future, the ZERS questionnaire presented can be integrated with other measurement tools to investigate other stakeholders' opinions. However, at this first stage, it was decided to analyze the opinion about the ethical reputation of zoos only in the category of young and adult visitors,

who do not represent all stakeholders but are among those who very easily can spread word of mouth about the reputation of a particular zoo.

However, a very important step was represented by the identification of the main drivers that can impact the reputation of zoos. Based on them, it will then be possible to customize ZERS, creating questionnaires with items adapted to analyze the opinions on the reputation of zoos—determined by the particular interests of each stakeholder—of different stakeholder categories. The results will provide important information to the zoo on what it has been able to communicate regarding its efforts for animal welfare, its work in biodiversity conservation, and its ability to implement effective educational projects. This can allow the individual zoo to figure out what aspect to improve. Furthermore, this will allow the zoo also to choose different strategies to satisfy that particular stakeholder category that has underlined a possible critical issue. This information can then be shared with other zoos to benefit the community of zoos as a whole.

Conclusions

ZERS is a tool that assesses and highlights people's perceptions about 12 drivers that can influence the reputation of zoos. Similar tools, such as Reputation Quotient $^{(SM)}$, are well established for the evaluation of the reputation of other corporations (Fombrun et al., 2000), but, to our knowledge, there are no similar tools to evaluate the reputation and ethical aspects of zoological institutions. Yet, nowadays, zoos are progressively under the scrutiny of public opinion, and many factors can negatively influence their reputation by offering an excuse to those who consider these institutions obsolete or a "nineteenth-century anachronism" (Stevens & McAlister, 2003).

In the development of the tool, many drivers that can influence the reputation of a zoo have been identified. Zoos must be very careful of their reputation to ensure that they thrive in the future as major conservation organizations, as a negative reputation may quickly lead to a reduction in the number of visitors and funding for conservation projects (Wilson, 1984; Gray, 2017). Reputation

can take a long time to build up and coalesce in people's minds, but research shows that it can be extremely difficult to change once formed (Wartick, 1992). This must be taken into adequate consideration, especially with regard to a negative reputation. Therefore, there is an urgent need to develop tools to analyze visitors' opinions on components that can affect the reputation of zoos.

So far, despite the vast literature on the reputation assessment of companies whose main objective is to improve their income, there is no research on the development of tools to assess the reputation of zoos. This is probably due to the fact that zoos—which have as their goals not only economic interests but also, and above all, the welfare of wild living beings, the protection of biodiversity, and the education of their visitors—are much more complex entities. ZERS can fill this gap and help these institutions to assess their ethical reputations. Zoological associations know very well how important it is for them to act ethically not only in the management of animal welfare but also in their actions and communication with all other stakeholders. For this reason, in 1995, the World Association of Zoos and Aquaria (WAZA) drew up its own ethical code, which has been continuously adapted and updated over the years and to which all its members must adhere.

ZERS can help zoological associations evaluate how much the public perceives the commitment of their members. At the same time, the use of ZERS can also enable individual zoos to highlight critical issues and implement strategies to improve them. By addressing them, zoos can not only increase people's trust and involvement in their biodiversity conservation efforts but also, by reflecting on measurable parameters, they are encouraged to operate as ethical institutions, "ethical arks" committed to advancing higher standards and practices towards all their stakeholders.

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2.2. Global response of conservationists across mass media likely constrained bat persecution due to COVID-19

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Abstract

Most people lack direct experience with wildlife and form their risk perception primarily on information provided by the media. The way the media frames news may substantially shape public risk perception, promoting or discouraging public tolerance towards wildlife. At the onset of the COVID-19 pandemic, bats were suggested as the most plausible reservoir of the virus, and this became a recurrent topic in media reports, potentially strengthening a negative view of this ecologically important group. We investigated how media framed bats and bat-associated diseases before and during the COVID-19 pandemic by assessing the content of 2651 online reports published across 26 countries, to understand how and how quickly worldwide media may have affected the perception of bats. We show that the overabundance of poorly contextualized reports on bat-associated diseases likely increased the persecution towards bats immediately after the COVID-19 outbreak. However, the subsequent interventions of different conservation communication initiatives allowed pro-conservation messages to resonate across the global media, likely stemming an increase in bat persecution. Our results highlight the modus operandi of the global media regarding topical biodiversity issues, which has broad implications for species conservation. Knowing how the media acts is pivotal for anticipating the propagation of (mis)information and negative feelings towards wildlife. Working together with journalists by engaging in dialogue and exchanging experiences should be central in future conservation management.

Introduction

Mass media represents one of the main news referring sources for people, and media framing plays a crucial role in shaping society's attitudes towards wildlife (Chong and Druckman, 2007; Gore and Knuth, 2009). In the digital era, reading news on the Web has become a regular habit for many people, and the information provided by mass media has gained the ability to reach a global audience within a very short time. Today, most newspapers produce an online version, offering unlimited coverage of breaking news worldwide. Moreover, social media has increased news visibility enormously. People share news on social media and thus act as news filters, often spreading and overemphasizing the most alarming news stories (Mammola et al., 2020; Nanni et al., 2020). Media framing may strongly shape public risk perception (Leiserowitz, 2005) and has become extremely important in promoting or discouraging public tolerance towards wildlife conservation, especially for species that ignite the human-wildlife conflict, such as large carnivores (Arbieu et al., 2021; Bombieri et al., 2018; McCagh et al., 2015; Nanni et al., 2020), spiders (Mammola et al., 2020, 2022a, 2022b), viruses (Evensen and Clarke, 2012), and bats (Cerri et al., 2021).

Bats have been identified as hosts of serious zoonotic diseases, including Nipah and Hendra virus, Rabies, and several Respiratory Syndromes (Schneeberger and Voigt, 2016). The connection with zoonotic diseases has considerable potential to negatively impact human perception of bats by evoking fear and intolerance among the public (Vaske et al., 2009; Wobeser, 2006), especially if risk communication is poorly contextualized and inadequately crafted (MacFarlane and Rocha, 2020). Negative perception of bats may be explained by an inborn fear for animals associated with the spread of diseases (Davey, 1994; Matchett and Davey, 1991; Prokop and Tunnicliffe, 2008; Ware et al., 1994; Whitaker and Douglas, 2006), as well as by the way in which information is framed by the mass media and by the scientific literature. A review conducted by Lopez-Baucells et al. (2018) ´ highlighted that half of the virological studies regarding bats framed them as a major concern for public health without providing evidence, while only 4% of such studies mentioned their importance for ecosystem functioning. In this

sense the scientific literature acts as a possible source of (mis)information for mass media and the information shared by the scientific literature may be replaced and amplified by the mass media, which also often frame bats as a serious threat to human health (Schneeberger and Voigt, 2016). The overabundant news relating to specific topics, such as bat-associated diseases, may lead to an overestimation of the risk posed by bats and, in extreme cases, may fuel direct persecution of these suspected disease reservoirs (Buttke et al., 2015; Guyton and Brook, 2015). A balanced and accurate communication about health risk involving bats is fundamental to both mitigate the spread of diseases and render conservation efforts for bats more effective (Crockford et al., 2018; Lopez-Baucells et al., 2018). Bats have key functional role,
and their conservation may improve ecosystem functioning, positively affecting economy (Boyles et al., 2011) and even human health, following the "One Health" concept (Decker et al., 2010).

A unique opportunity to globally assess the importance that communication plays for wildlife conservation was provided by the novel zoonotic coronavirus (COVID-19), that at the end of 2019 was isolated in China, and which underwent a rapid global spread between January and March 2020, with marked social and economic effects (World Health Organization, 2020). Even though the origin of COVID-19 is still debated, shortly after the onset of the COVID-19 outbreak, several studies suggested bats as the likely natural reservoir and origin of the virus (Lu et al., 2020; Wu et al., 2020; Xu et al., 2020; P. Zhou et al., 2020; Zhu et al., 2020). This information was replayed and globally spread by the mass media during the first months of the pandemic, possibly raising public anxiety and intolerance towards bats (Lu et al., 2021; Rocha et al., 2020).

We studied the effects of information on bats delivered by the media to assess how quickly a biased negative representation of wildlife by global press may undermine conservation efforts. We gathered global media reports on bats from before and during the pandemic across 26 countries and in 7 languages. We asked the following questions:

1. What is the content of the information of each bat-related media report?

2. How has the information contained in media reports changed throughout the first months of the COVID-19 pandemic?

Methods

Media news retrieval

Online media reports regarding bats were collected across the globe for the period 2018–2020, using seven languages, i.e. English, Spanish, Chinese, French, Portuguese, German, and Italian. Specifically, we analyzed reports in 26 countries, i.e. China, India, Pakistan, United States of America, Canada, United Kingdom, Italy, Spain, France, Portugal, Germany, Austria, Ecuador, Peru, Argentina, Costa Rica, Brazil, Australia, New Zealand, Philippines, Democratic Republic of Congo, Namibia, Kenya, Ghana, Senegal, and South Africa, covering all six continents on which bats occur. We adapted the methodology used in Nanni et al. (2020) and Mammola et al. (2020) for retrieving online media reports on bats. The online search was conducted via the advanced Google search tool, using "bats" or the corresponding translations as a keyword, and adjusting the language and country accordingly. We specified the temporal interval of the research, i.e. one year at time (e.g. 1/01/2018 to 31/12/2018) using the 'Custom range' tool. For each year, via Google News we collected the first 50 bat-related news reports, as for the majority of countries no more news were available. We excluded non-pertinent reports (e.g. those related to batman, bat robots, or sport bats). Reports from online magazines were included, as well as those from blogs or YouTube videos if they represented television news from newspapers.

Data extraction

A content analysis was performed (Krippendorff, 2018). For each media report, we extracted or derived the following information: (a) title, (b) publication date, (c) newspaper name, (d) newspaper circulation ('local', 'national' or 'worldwide'), (e) topic of the news, (f) sensationalism, (g) presence of pro-conservation messages, i.e. messages promoting bats conservation and safeguard, and (h) bat species or families mentioned (if any).

We classified newspaper circulation as 'local' if their total circulation (paper + online) was below 50,000 copies, and as 'national' if it was above 50,000 copies, searching the total circulation on each newspaper webpage and cross-checking this on the Wikipedia newspaper trend page. To define newspaper circulation as 'worldwide' we used the World Press Trends 2016 News (Milosevic, 2016).

Concerning the topic of the news, we defined the following categories: (i) 'bat-associated disease', if the report was about diseases transmitted by bats to humans (articles about wet markets were included in this category); (ii) 'persecution', if the news focused on bats killing or persecution; (iii) 'dead bats', if the news main topic was about bats found dead for natural or unknown causes; (iv) 'science communication', if the news was mainly about research findings, new species discovered, or if it was an interview with a scientist; (v) 'others', for topics not fitting into the previous categories. Although that same report may encompass several of the topics above, we decided to focus on the main topic of each one which was usually expressed in the title. For reports classed as 'persecution', we created an identifier for each unique event (ID_ persecution) and collected the year when the event occurred to be able to recognize each unique persecution event.

To assess a media report as sensationalistic, we evaluated the title, subheading, and main text of each media report. Following the definition of 'sensationalism' by Uribe and Gunter (2007): "a characteristic of the news-packaging process that places emphasis upon those elements that could provoke an effect on the human sensory system", we considered a report as sensationalistic if it contained at least one markedly negative word as: "horror", "horrific", "nightmare", "evil", "scary", "terror", "terrifying", "terrorizes", "frightening", "alarm", "panic", "attack", "devil", "hell", "killer", "terrible", "disturbing", "creepy", "disquieting", "dreadful", "awful", "monster", "invasion", "under siege", "plague", "petrifying", "spookier", "filthy". However, we did not classify a report as sensationalistic if such words were used ironically or rhetorically to express the opposite meaning, (e.g. "Are bats really awful creatures?", or "Is all this terror for bats necessary?"). To standardize the data mining strategy among different authors in charge of

different countries and languages, we prepared a general protocol for retrieval and classification of information on reports. Moreover, the entire final database was checked for consistency by the first author to assess uniformity in the classifications.

Finally, we assessed the occurrence of pro-conservation messages by checking if each media report: (1) mentioned the importance of bats for ecosystems; (2) mentioned the extinction risk of bat species or bats in general; (3) gave motivations for safeguarding bats; (4) gave advice on how to safeguard or assist bats.

Data on COVID-19

We recorded information on the spread of the COVID-19 pandemic from January to July 2020 across the 26 countries investigated (Dong et al., 2020). Specifically, for each country we collected: a) number of new infections every 15 days; b) number of total cases until July 31st; c) number of total deaths until July 31st; d) number of residents; and e) date of the first exponential growth of the epidemic curve, i.e. the date on which each country started to experience widespread transmission inside the community, based on the data collected by Ficetola and Rubolini (2021). We used this information to define whether each report was published before or after the first exponential growth of the epidemic curve.

Data analysis

We conducted all analyses in R (R Core Team, 2021). To assess whether the pandemic affected the media framing of bats, we built three generalized linear mixed models (GLMMs) with a binomial error distribution and tested the significance of independent variables with a likelihood ratio test (Bolker et al., 2009). In all models, we included the country of search as random factor. In the first model, we tested whether news on bat-associated diseases became more frequent after the emergence of COVID-19 in January 2020. We used the presence/absence of news focused on bats as disease vectors as dependent variables, while the year of publication and the newspaper circulation as independent variables. We used orthogonal contrasts (Field et al., 2012) to assess

if the frequency of news describing bats as disease transmitters differed between 2020 and the pre-covid period (i.e. 2018 + 2019), and then between 2018 and 2019. Furthermore, to assess differences associated with the newspaper circulation, we subsequently performed a Tukey post-hoc test among the levels (international, national, local), using the function glht of the package 'multcomp' (Hothorn et al., 2008). In the second model, we related the presence/absence of pro-conservation messages (dependent variable) to the year of publication and the newspaper circulation (independent variables). We designed the third GLMM model to verify if sensationalistic framing increased during the COVID-19 pandemic. We used the presence/absence of sensationalism as dependent variable and year and newspaper circulation as independent variables.

We then focused on what happened in the year 2020 (N = 1160), namely during the pandemic period, to assess how the spread of bat- related news and pro-conservation messages varied according to the diffusion of COVID-19 in each country. We built two GLMMs with a binomial error distribution, both having the logarithm of the number of cases, logarithm of incidence, newspaper circulation level and the variable "pre/post exponential" as independent variables, the latter defining whether a given report occurred before or after the first exponential date of the epidemic curve. In the first model, we used the presence/absence of a bat-associated disease in the report as the dependent variable, while in the second model we used the presence/ absence of pro-conservation messages as the dependent variable.

Finally, using a Chi-squared test, we verified if the number of persecution events increased after the emergence of COVID-19. Given that for the year 2020, we only considered January/July (i.e. 7 months), we weighted the number of yearly events by the number of months for which the information was available.

We graphically explored the content of reports with barcharts using 'ggplot2' (Wickham, 2016). Using density plots, we explored the temporal distribution of bat-associated disease reports, pro-conservation messages and new COVID-19 infections, by computing a kernel density estimate with a 1.5 bandwidth adjustment (Wickham, 2016).

Results

We collected a total of 2651 reports regarding bats, published between January 2018 and July 2020 from 26 countries (Fig. 1).

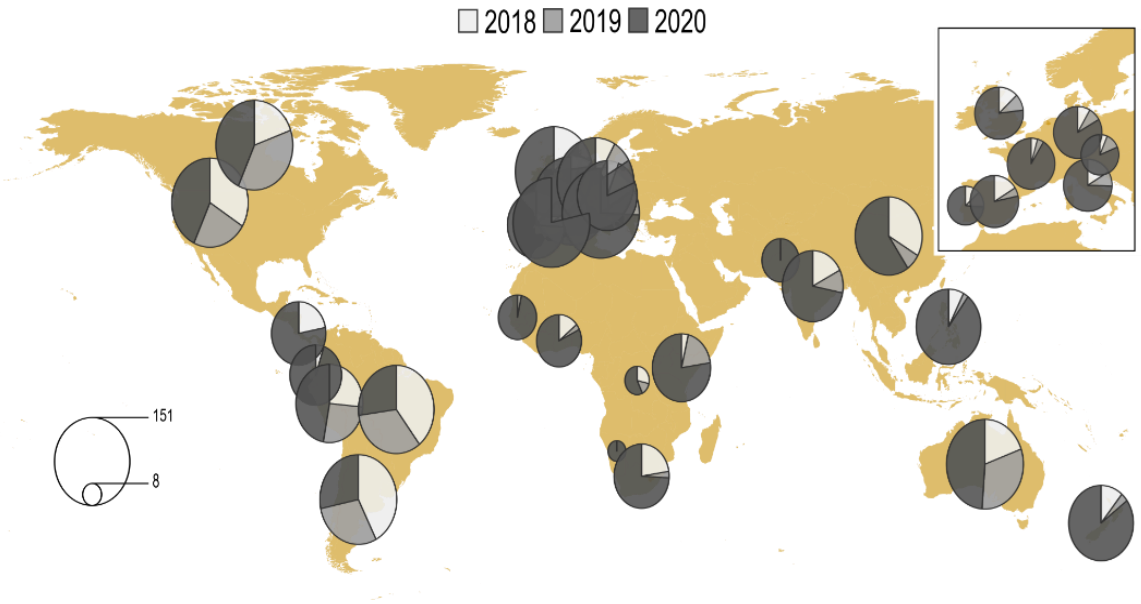


Fig 1. Yearly proportion of reports on bat-associated diseases

We identified a total of 21 single events of persecution towards bats described in the news, with an increase in 2020 compared with previous years (χ^2_1 = 7.4, P = 0.006). In African countries, the annual number of online published reports regarding bats was less than 50, especially before 2020. Reports were published in 1104 different online newspapers, mainly at the national level (71.1%, n = 1885), followed by local (22.7%, n =601), and worldwide levels (6.2%, n =165). The majority of reports focused on pathogenic elements of potential zoonotic risk identified in bats (42%, n = 1113), 'others' (35,2%, n = 934) and science communications (18.3%, n = 484), while few reports focused on dead bats or persecution (3.4%, n = 89, and 1.2%, n = 31 respectively). The category 'others' included, for example, news regarding events organized for the public, bat-focused projects, white-nose syndrome, bats found in dwellings, bat tourism and eating bats, as well as summary reports on the general status of bats and their ecosystems and

impacts of infrastructures. Considering the total number of reports in each country, reports regarding bat-associated disease were higher in Africa (between 46.7% and 81.1%), Asia (between 43.4% and 71.4%) and Central-South America (between 55.9% and 66.7%), compared with North America (between 43.4% and 71.4%), Oceania (between 25.5% and 27.3%), and Europe (between 15.9% and 40.7%).

The frequency of reports describing bats as disease transmitters was significantly different across years (GLMM: $\chi^2_2 = 301.7$, $P < 0.001$). Orthogonal contrasts showed that reports describing bats as disease transmitters were much more frequent in 2020, the global outbreak year, than in 2018 and 2019 ($\chi^2_1 = 295.1$, $P < 0.001$). Furthermore, in 2019 we found slightly fewer reports on this topic compared with 2018 ($\chi^2_1 = 8.3$, $P = 0.004$). Differences between newspaper circulation levels were detected ($\chi^2_2 = 10.7$, $P = 0.005$), with fewer reports describing bats as disease vectors in international newspapers compared with both national and local newspapers (Tukey's post hoc: both $P \leq 0.01$), while we did not detect differences between national and local newspapers ($P = 0.956$). The variance of the random effect for country of search was 0.52 (SE =±0.72). The majority of news had no sensationalistic components (95.6%, n = 2534), and rate of sensationalism was constant over the years ($\chi^2_2 = 2$, $P = 0.36$).

The frequency of pro-conservation reports was significantly different across years and newspaper circulation categories (GLMM: $\chi^2_2 = 40.7$, $P < 0.001$ and $\chi^2_2 = 9.3$, $P = 0.01$, respectively). Orthogonal contrasts showed fewer pro-conservation messages in the media in 2020 compared with previous years ($\chi^2_1 = 40.4$, $P < 0.001$). Tukey's post hoc test showed that reports containing pro-conservation messages were more frequent in local newspapers compared with national ones ($P = 0.005$), while no differences were detected between national and international or local and international newspapers ($P = 0.994$ and $P = 0.157$, respectively). The countries where more than half of the total news published contained pro-conservation messages were Germany (78%, n = 117), Canada (64.7%, n = 97), United Kingdom (62.9%, n = 95), Spain (59.3%, n = 89), New Zealand (55.7%, n = 59), Australia (55.3%, n = 83), and France (52.4%, n = 76).

Focusing on 2020, the frequency of disease transmission reports did not follow the epidemic course of each country (Fig. 2). Indeed, we found no correlation between the date of the first exponential growth and the probability of disease transmission reports occurring ($\chi^2_1 = 0.3$, P = 0.6). Conversely, almost all countries registered a first peak in the number of disease-related news at the beginning of 2020, during the diffusion of the epidemic in China ($\chi^2_1 = 0.3$, P = 0.6; Fig. 2). We observed an increase in pro-conservation news during 2020, which occurred consistently after the onset of the exponential growth of the epidemic curve in each country ($\chi^2_1 = 10.2$, P = 0.001; Fig. 4). The onset of the exponential growth was the only variable showing a significant relationship with the probability of finding pro-conservation reports.

Fig. 2. Comparison between the spread of both news reports on bat-associated diseases (grey) and the COVID-19 pandemic (purple) in each country in 2020. Namibia was excluded because no reports on bat-associated diseases were located. We considered the temporal trend of both news on bat-associated disease and emerging cases of COVID-19, every 15 days. The cumulative curves for the media news and COVID-19 cases were estimated with a kernel density estimation. In the majority of countries, the first peak of news on bat-associated diseases news coincided with the first peak of the epidemic in China, regardless of whether the epidemic had arrived (χ^2_1 = 0.3, P = 0.6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

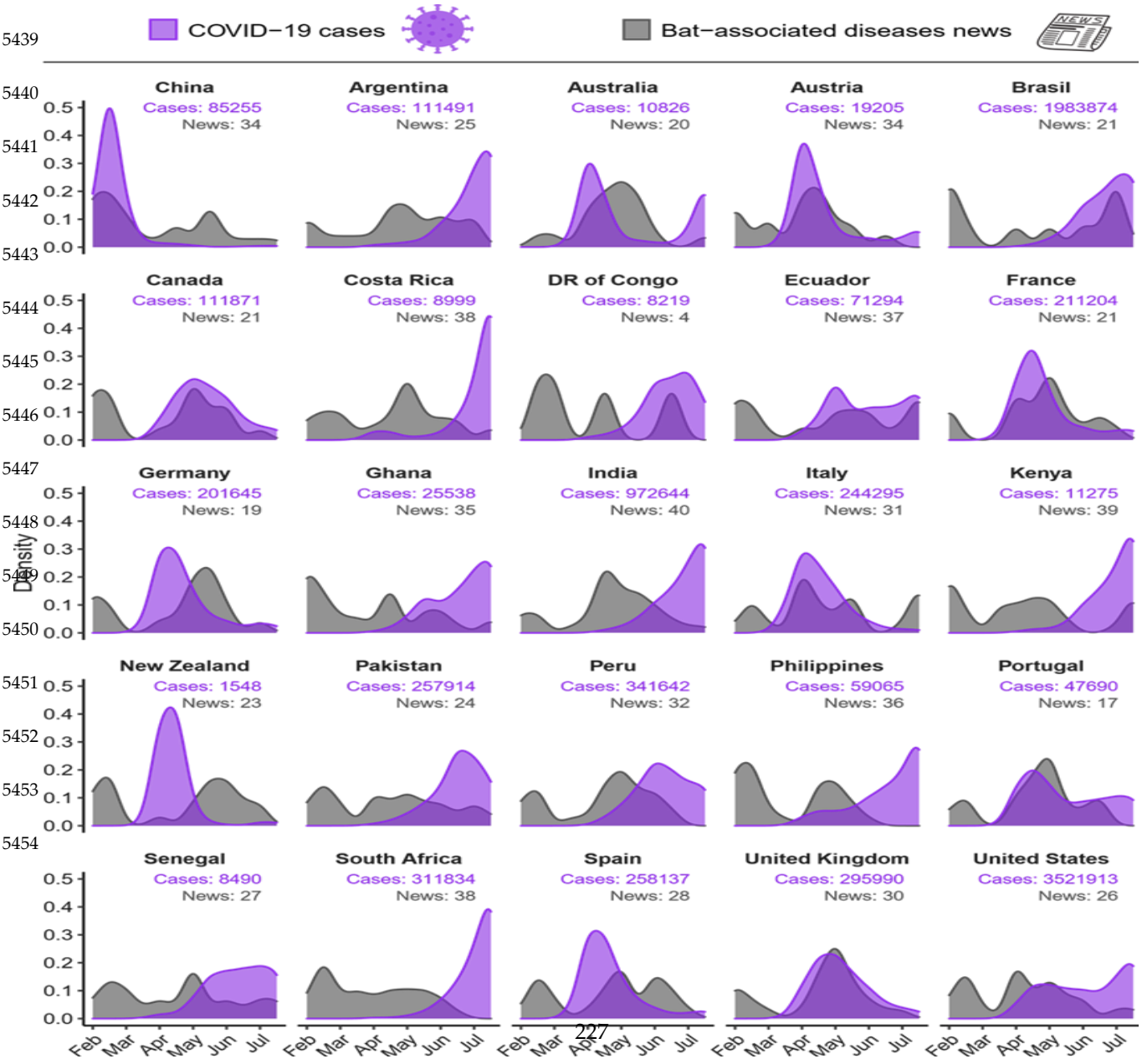
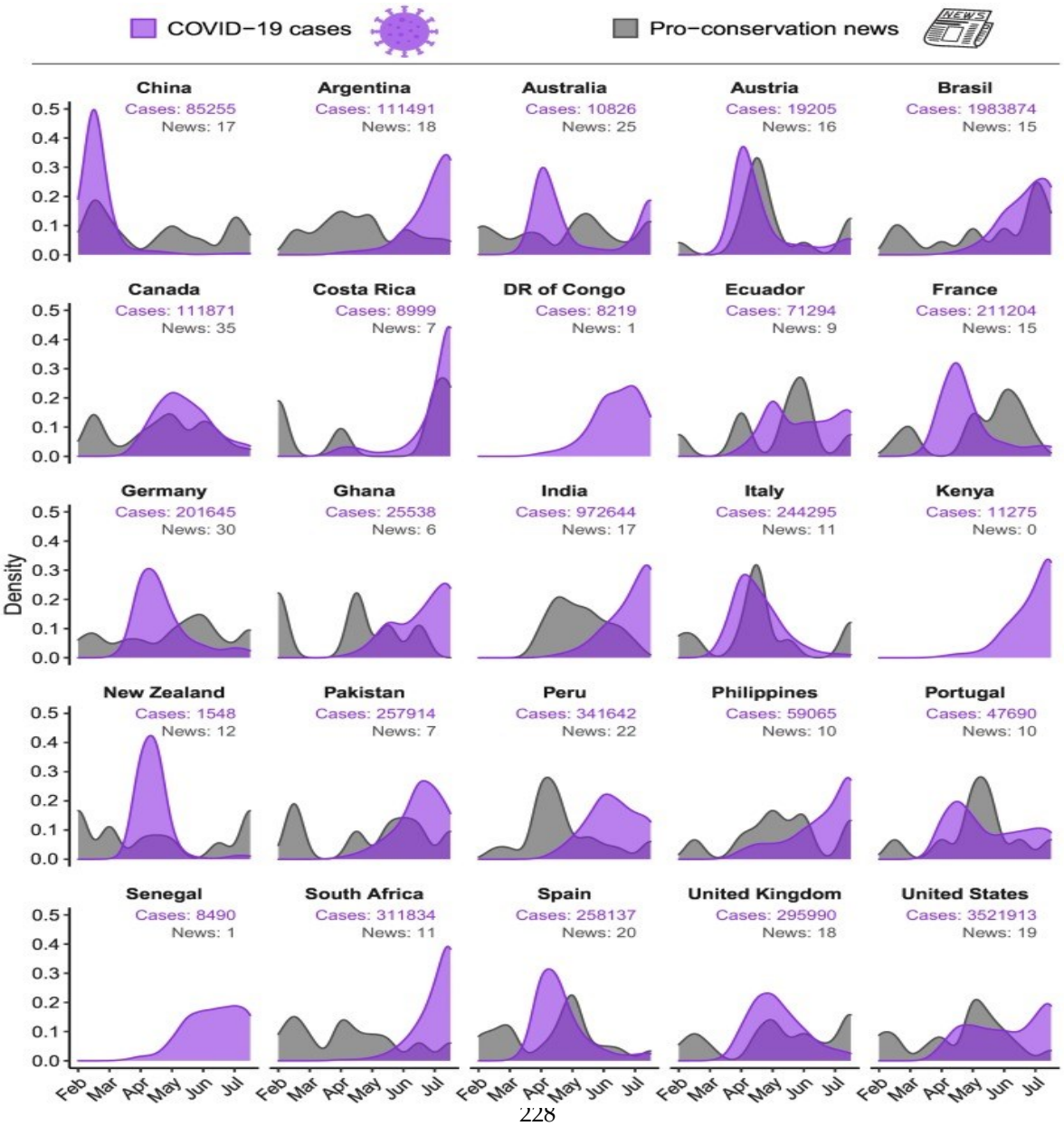


Fig. 4. Comparison between the spread of news reports containing pro-conservation messages (grey), and the COVID-19 pandemic (purple) in each country in 2020. Namibia was excluded because no pro-conservation reports were located. We considered the temporal distribution of both pro-conservation media reports, and emerging cases of COVID-19, every 15 days. The cumulative curves for pro-conservation news and COVID-19 cases were estimated with a kernel density estimation. Pro-conservation reports were significantly more frequent after the first exponential growth of the epidemic curve in each country ($\chi^2_1 = 10.2$, $P = 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Bat species had different popularity in the media . The species with more than 25 citations were: *Desmodus rotundus, Myotis lucifugus, Pipistrellus pipistrellus, Chalinolobus tuberculatus, Acerodon jubatus, Rhinolophus errumequinum, Rhinolophus hipposideros, and Pipistrellus pygmaeus*. The common vampire bat (*D. rotundus*) and the giant golden-crowned flying fox (*A. jubatus*) where cited by newspapers all around the world despite their limited geographical occurrence (Central-South America and Philippines, respectively). The other species were mainly cited by newspapers of countries in which those species normally occur. In the case of the two Rhinolophus species, which were found to host the closest - known - relative of SARs-CoV-2, they gained visibility outside their geographic range after the emergence of the pandemic. However, most of the time media news just mentioned the genus or family, without giving the exact species name. The most cited families were Pteropodidae (n = 156), Rhinolophidae (n = 54), and Vespertilionidae (n = 34).

Discussion

News plays a major role in the human perception of wildlife and biodiversity. Most people have little direct experience with wildlife, and the mass media often becomes the means by which people connect with nature, thus their importance on transmitting reliable information to help species conservation. Our interest was to identify how mass media around the world shaped the risk perception on bats by humans. We found that events of persecution towards bats increased after the COVID- 19 outbreak, possibly driven by the raise in the media representation of bat-associated diseases. As demonstrated in other studies, news exposure provokes a social amplification of risk associated with wildlife (Gore et al., 2005; Gore and Knuth, 2009). However, the action of conservationists in disseminating pro-conservation messages immediately after the surge in reports on bats as disease transmitters, may have helped to reduce the public's negative perception of bats due to COVID- 19. According Harcup and O'Neill (2017) news delivery satisfy the ‘surprise’ and the ‘follow-up’ requirements (among others), stories having an element of surprise and/or contrast, as well as stories that introduce new elements on

subjects already in the news, were preferred in the media dialogue. Thus, journalists likely received messages delivered by conservationists as an opportunity to fuel the media dialogue and include them into the news. Our results provide guidance for responding and contributing effectively to media coverage, a fundamental component of efforts for wildlife conservation (Table 1).

5536 Table 1. Transparent and correct communication: the role of different stakeholders

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STAKEHOLDER	SHOULD
 JOURNALISTS	Use evidence-based language. Consult scientific experts to debunk misinformation. Use informative images or intuitive graphical abstracts
	Avoid eye-catching/negative images or sensationalistic words
	Emphasize the positives aspects of bats (ecosystems services such as pollination and seed dispersal, consumption of pests, etc.) and their important role in the extended food web
	Avoid reporting or mentioning myths/urban legends about bats
	In debunking hoaxes and misinformation, use effective communication strategies to minimize unwarranted effects (e.g., the Truth sandwich approach)
	Promote sections in the media that talk about the natural history of species
	Give helpful information about how bats diseases can be transmitted and how avoid it
 CONSERVATIONISTS	Use high profile species (with appealing or charismatic traits) as symbols or ambassadors for conservation campaigns
	Explain risks with understandable examples (people make subjective judgments about the severity and likelihood of a risk, e.g. zoonotic diseases); use evaluable comparisons to other events to explain the maths ("e.g. getting rabies from bats is as unlikely as ..."
	Avoid the use of technical jargon or graphics that are not easily understandable to non-scientists
	Develop collaborations with relevant press agencies and pre-prepare positive/informative communications for ciclic events that likely gain media attention (wolves, carnivores and sharks attacks, snake occurrence, spiders bites, insects spreading is etc.) to be delivered when consideration raises
	Mediated messages toward locally direct experiences with nature
	Promote classes and lectures to key professionals indirectly involved with bats and/or its diseases, such as journalists, veterinarians, physicians and nurses, to demystify how bat diseases are transmitted and to how avoid them, as well as the importance of bats conservation and the role of these professionals to help in it
	Expand scientific dissemination resources, using different social media, podcasts, radio, tv and messaging apps to promote bats conservation
	Expand science dissemination in schools by promoting educational conferences for teachers and meetings/activities with students
	Give concrete solution rather than just describe problems
	Use media strategy to disseminate conservation messages
	Mitigate conflicts listening and conversing with all stakeholders involved, taking into account their necessities
 PUBLIC	Check the correctness of information read (fact-checking) by developing a critical sense, checking the author and source of information, verify the date, using tools such google images to search for the original upload of images or video used in the news.
	Read all the content of the journalistic report.
	Follow experts on social media or trustable sources.
	Contact experts if major doubts are available
	Ask local governments to provide the correct information about difficult subjects (eg. zoonosis).
	Check more than one media source
	Be aware of emotion appealing newspaper reports. If a report make you scared probably it is has been designed to do that

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Increase in bat-associated diseases news and bat persecution

A large proportion of the collected reports focused on bat-associated diseases, with a significant increase in 2020 compared with the previous years . However, the number of reports regarding bat- associated diseases was not correlated with the spread of the epidemic curve in each country. Instead, in most of countries, a first peak in the amount of news on bat-associated diseases was registered during the spread of COVID-19 in China, even if in those countries the epidemic had not yet arrived (Fig. 2). This result suggests that the first COVID-19 outbreak in China was the main driving force for the worldwide media. Certainly, following the spread of the virus in China, news linking bats to COVID-19 were frequently in the spotlight of the global press. Many news denounced an increase in human intolerance for bats following the COVID-19 pandemic (e.g. in India https://cutt.ly /OxfOU9W; or in Singapore https://www.tnp.sg/news/singapore/mor e-calls-acres-feb-COVID-fear-led-bat-publicity; see also Manenti et al., 2020) and, according to our results, news may have contributed to increase persecution events in 2020. Even if it is possible that prior to the pandemic bat persecution events did not gain mass media attention, this apparent rise in fear and intolerance towards bats, which in extreme cases ended with direct persecution, was likely related to the media overrepresentation of bat-associated diseases and the spread of misinformation in the media during the first months of the COVID-19 pandemic. Similarly, Lunney and Moon (2011) found that media attention on zoonoses without supporting evidence on disease transmission risks increased animosity towards bats in Australia. Undoubtedly, much of the public understanding of infectious diseases comes from information released by the mass media (Evensen and Clarke, 2012).

Attractive topics spread rapidly across the globe in the media, and effective conservation messages should be equally fast to anticipate the diffusion of misconceptions and negative feelings among the public to avoid direct persecution of wildlife. Working together with journalists by engaging in dialogue and exchanging experiences should be central in any conservation program as well as advise the public on how to handle the information ecosystem, for example checking the correctness of reports and avoiding to share dis- or mis-information on

social media. The new information ecosystem poses a real challenge to conservation, funds for communication campaigns should be implemented given the wide scale impact they may have, as highlighted by our work. We provide some hints on how communication messages should be designed and promoted by conservationists and journalists and how public should navigate through the information ecosystem. Future studies should test the effectiveness of efforts undertaken by conservation project to promote the public outreach and mass media coverage of wildlife. Foster multidisciplinarity by including sociologists, anthropologists and communicators in conservation planning is pivotal to achieve conservation goals.

All authors read the text, provided comments, suggestions and corrections, and approved the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement The database supporting the study is available in Figshare (https://figshare.com/articles/dataset/db_bats_news_xlsx/19778812).

Credit authorship contribution statement: Conceptualization: VN; Data collection: AC, NM-H, ALS, EL, CM, RA, MS, SH, ZZ, JZ, RM, VN; Data management: VN; Data analysis: GFF, VN, RM; Data visualization: VN, SM; Writing (first draft): VN; Writing contributions: NM-H, RM, GFF, EM-dM, ALS; English revision: DC

All authors read the text, provided comments, suggestions and corrections, and approved the final version.

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2.3 Public perception of the consequences caused by the COVID-19 pandemic on zoological institutions: The Italian case.

Simply Summary

The present work presents the results of a survey aimed at investigating what the people perceived about the difficulties zoo workers faced in Italy during the COVID-19 pandemic. Data collected from 588 respondents indicate that people believe that zoos and aquaria play a relevant role in wildlife conservation and scientific research and a significant role in environmental education. After the COVID-19 outbreak, zoo workers abruptly faced considerable economic difficulties and problems in maintaining the welfare of the hosted animals. As millions of people stayed indoors, zoo workers had to reinvent their education activities and find new strategies to maintain a relationship with their visitors and for fundraising. Understanding what people perceived about these zoological institutions' difficulties is essential to find new strategies to engage people and support them if something similar happens again.

Abstract

In late 2019, the World Health Organization declared the outbreak of a global pandemic COVID-19, a disease caused by a new variant of Coronavirus, the 2019-nCoV. On March 2020, the World Health Organization declared COVID-19 a pandemic disease, and from that moment, the COVID-19 pandemic significantly restricted human activities worldwide. The Italian Government was the first to order a nationwide lockdown limiting the movement of people as a preventive measure. Suddenly zoos were forced to close to the public. Despite this, the zoo workers continued their work, facing unexpectedly complex difficulties in sustaining the zoos financially and maintaining high animal welfare standards. Months later, zoos were reopened

with several limitations to the public. To evaluate the awareness of the public about the difficulties zoo workers had to face during COVID-19, we administered a survey to zoo visitors and online to the general public. The results of 588 respondents show zoos and aquaria are considered to play a very important role in wildlife conservation (Very Relevant 48.8 % and Relevant 38.8 %), and wildlife research (Very Relevant 55.4 % and Relevant 34.9 %). The respondents perceived as very relevant (RII= the negative economic consequences that zoos and aquaria had to face.

Background

At the beginning of 2020, Italy was caught by surprise by outbreaks of atypical pneumonia cases. Similar cases had already been registered in the People's Republic of China and reported – on 31 December 2019- to the WHO China Country Office by the Municipal Health Commission of Wuhuan, the capital of Hubei Province (National Health Commission of the People's Republic of China, 2021; WHO, 2020a). The etiological agent was promptly isolated in patients' blood samples, throat swabs, and lung fluids and identified as a new virus of the Coronaviridae family (Lu et al., 2020). The World Health Organization previously named it 2019-novel-Coronavirus, 2019-nCoV (WHO, 2020c, 2020b), but later — on 11 February 2020 — after further genetic analysis, the International Committee on Taxonomy of Viruses (ICTV) renamed it "severe acute respiratory syndrome coronavirus 2" and referred to it by the acronym SARS-CoV2. A month later, on 11 March 2020, WHO assessed that COVID-19, the disease caused by SARS-CoV2, could be characterized as a pandemic (WHO, 2020d).

Before that WHO declared COVID-19 a Public Health Emergency of International Concern on 30 March (WHO, 2020c), the Italian Government — which had already limited the movement of people starting from 23 February 2020 (D.L. 6/2020) — ordered a nationwide lockdown (D.P.R. of 11 March, 2020) due to exponential increase in cases. Then, when the infection curve dropped, the restrictive measures relaxed (starting from 14 May 2020), and in June 2020 the movement between Italian regions was again permitted. But, from the beginning of October 2020

until the spring of 2021, the increase in the number of cases imposed again some restrictions (e.g., compulsory masks, curfew at 6 p.m., etc.) and the partitioning of the country into several risk zones with a ban on movement in the highest risk areas. In addition, nationwide closures were imposed during the holiday periods of Christmas 2020 and Easter 2021.

COVID-19 outbreak was immediately linked to a vast Chinese wet market selling live animals in Wuhan because the first patients were workers or visitors of this wet market (Hui & Zumla, 2019; Lu et al., 2020; Zhu et al., 2020) and bats were soon suspected to be the reservoir of this new virus, with pangolins or civets cats as a potential intermediate reservoir (Hui & Zumla, 2019; Luan et al., 2020; Xiao et al., 2020; Ye et al., 2020). Most emerging infectious diseases of humans, which are significantly affecting public health and global economies, are caused by viruses originating from non-human animals through zoonotic transmission; and, this event is known as spillover (David M. Morens et al., 2004; Jones et al., 2008; Moreno et al., 2015). Bats, due to their peculiar immune system, shaped by a benign virus-host relationship evolution (Subudhi et al., 2019), host many viruses which severely affect other mammals, including humans, but that are nonpathogenic for bats (Shea et al., 2014). The analysis of the complete single-stranded of viral RNA genome sequence (29,903 nucleotides) confirmed the suspects (Gorman, 2020). It revealed that SARS-CoV-2 is phylogenetically close (89.1% nucleotide similarity) to a group of SARS-like coronaviruses, the Betacoronavirus, previously found in bats in China (Wu et al., 2020).

Italy, the *Rhinolophus ferrumequinum* was suspected to be a potential primary reservoir of the virus, and scientists were afraid that they could infect secondary reservoirs, such as domestic, determining a specific risk of SARS-CoV2 infections (Buonocore et al., 2020). Fears that not only wild animals but also pets could spread SARS-CoV2 seemed to be confirmed when in late February 2020, a dog and a cat in Hong Kong tested positive for the virus (Hosie et al., 2021; Parry, 2020). Although the dogs have never developed clinical signs, cats presented COVID-related symptomatology (Gollakner & Capua, 2020). In addition to preliminary studies that demonstrated human-to-cat transmission (Hosie et al., 2021), and cat-to-cat transmission of

SARS-CoV-2 (Shi et al., 2020), in 2022 a genetic study supported the hypothesis of cat-to-human transmission, specifically to a veterinarian, in Thailand (Sila et al., 2022). Since the beginning of the COVID-19 outbreak, the fear of SARS-CoV2 transmission has led to many restrictions for zoological facilities.

Before the COVID-19 pandemic, zoos worldwide welcomed more than 700 million visitors each year (WAZA), and for many people, these institutions represent the only connection with Nature. After the COVID outbreak, the zoos and aquaria were closed to the public. They had to deal with multiple issues, such as unexpected loss of revenues and difficulties in animal management. Guidelines were issued by the Italian Society of Zoo and Wildlife Veterinarians and the European Association of Zoo and Wildlife Veterinarians to ensure the safety of zookeepers during the daycare of the animals (Lecu et al., 2020). In Italy, zoological institutions are private companies that rely entirely on ticket revenues, and due to population restrictions of movement, these zoological institutions have been exposed to the risk of being less able to care for the animals entrusted to their care (Bandoli et al., 2021). Also, their educational programs had to rethink their educational programs, starting e-learning programs (Bandoli et al., 2021). In Italy, the COVID-19 pandemic highlighted the vulnerability of these private institutions which, unlike other activities, work with animals and cannot be closed down overnight. Animals require constant care, routine feedings, enrichment and veterinary care, and sometimes, complicated health support systems. During the COVID-19 pandemic, the lockdown and many restrictions on population movement kept visitors away from zoos, and aquaria had to rethink their approach to funds raising. Social media was a powerful means of communicating at a distance with people who may be interested in supporting the zoos, and, for many, online fundraising became a key source of income during COVID-19 lockdowns and zoo closures (Ryder et al., 2021).

After the outbreak of COVID-19, many studies have been conducted to analyze changes in animal behavior caused by the absence of zoo visitors, and the mixed outcomes showed that such assessments require a species-specific approach (Carter et al., 2021; Williams et al., 2021). Several studies investigated the perception of workers on the difficulties and challenges they

faced during the COVID-19 pandemic (Fine et al., 2022). However, little research was focused on the public's perception of the difficulties and challenges zoological institutions faced during the COVID-19 pandemic. This paper presents a survey to assess the level of people's awareness of the difficulties zoological institutions have faced due to the pandemic.

Method

The Questionnaire

The questionnaire was designed to assess the general public's perception of the consequences of the lockdown and the periods of restrictions that followed after the COVID-19 outbreak. It was created by the ethicists of the Ethics Laboratory for Veterinary Medicine, Conservation and Animal Welfare of Padua University with the collaboration of veterinarians, zoologists, a sociologist, and a psychologist. The first version of the questionnaire, with 82 items, was initially created in November 2020 and uploaded to the online LimeSurvey platform for a pilot study. The link to the questionnaire was then diffused through social media as a pilot study. Only 89 people compiled the questionnaire, and several did not complete it. Through an iterative process among the team members, the items were reformulated and reduced in number so that the questionnaire could be compiled in less time. The final version of the questionnaire consists of three main sections and contains 26 items. The first section includes 6 items to assess the respondents' familiarity with zoos or aquaria and their opinion on the role of these institutions in wildlife conservation, research, education, and hosted wild animals' welfare. The second section, consisting of 13 items, investigates the respondents' opinions on the consequences of the pandemic on the role of zoos and aquaria, their workers, and the animals. The final third section contains 6 items to assess the demographic characteristics of the respondents and a last item that allowed us to understand whether the respondent filled out the online Survey or the paper survey administered in the zoos or aquaria.

A 4-point Likert scale ranging from "Strongly Agree", "Agree", "Disagree", and "Strongly disagree" was used to assess the respondents' opinions on statements regarding the consequences of the COVID-19 pandemic on zoological institutions. While a scale ranging from "Very relevant", "Relevant", "Slightly relevant", and "Not at all relevant" was used to measure opinions on the roles played by these institutions. Finally, for the item investigating the respondents' view on the grade of consequences of the COVID-19 pandemic on zoological institutions, a scale ranging from "Very significant consequences", "Significant consequences", "Insignificant consequences", "No consequences" was applied. For all items, it was also possible to choose the answer "I don't know" if the respondent had no opinion on that topic.

The questionnaire administration and data collection

The questionnaire administration was conducted in two ways: online and in person, with a paper-based questionnaire administered directly by researchers to visitors of Giardino Zoologico di Pistoia, Oltremare in Riccione, and the aquarium of Cattolica. The questionnaires were administered in all the zoological institutions, for approximately seven hours per day, between August and December 2021, from 10 a.m. until closing time. The potential participants were chosen by random sampling. After providing information on the purpose of the research and specifying that it was a voluntary and anonymous questionnaire, the researchers asked each potential respondent for verbal consent to participate in the Survey. Furthermore, to ensure anonymity, no personal data that could link the questionnaire to the respondent's identity in any possible way were collected. Participants could choose to fill out a paper questionnaire or access the online questionnaire with their mobile phone by scanning a QRcode. The online administration was done using a simple, quick, and anonymous online survey tool: LimeSurvey. The link to the Survey and the QRcode were also shared on media.

The Lime Survey was active from June 2021 to November 2021.

Statistical analysis

Data paper-based questionnaires were manually registered in an Excell sheet and combined with the data downloaded from LimeSurvey. After data screening (checking for missing data, unfinished questionnaires, etc.), the 4-Point Likert Scale answers were converted into numeric values: Strongly agree/ Very relevant/Strongly interested/ Very significant consequences = 4; Agree/ Relevant/interested/ Significant consequences = 3;
Disagree/ Slightly relevant/Slightly interested/ Insignificant consequences = 2; Strongly disagree/ Not at all relevant/Not at all interested/ No consequences = 1.
To rank the opinions of respondents according to their relative importance, the RII relative importance index was used in this study.

The RII of each item was determined using the following formula

$$RII = \frac{\sum W}{A \times N}$$

Where N is the total of the respondents, A is the highest weight, and W is the weight respondents gave to each factor from 1 to 4. W is calculated as the sum of n respondents selecting a response point (from 1 to 4) multiplied by the point's value.

RII values are then transformed into four levels of importance: high = $0{,}75 \leq RII \leq 1$; medium-high= $0{,}50 \leq RII \leq 0{,}75$; medium- low= $0{,}25 \leq RII \leq 0{,}5$; low= $0 \leq RII \leq 0{,}25$. A higher RII value shows that that item is more relevant to respondents.

Additionally, descriptive statistics were performed to review the collected data.

Results

One hundred and seventeen respondents took part in the Survey. After eliminating incomplete questionnaires, 588 questionnaires were analyzed to investigate the opinion of the respondents.

Of the 688 participants, 365 (62.1%) were females, 214 (36.4%) were males, 7(1.2%) preferred not to say and 2 answered "other". The participants' age ranged from 18 to 87 years (mean ± SD = 35.76 ± 11.85 years). The 25.7% (n=151) of the participants were aged 18–25 years, 62.6% (n=368) were aged 25–50 years, and 11.8 % (n=69) were aged over 51 years. Regarding the educational level, 48.6% (n=286) of the participants had a university degree or post-university degree, 41,5% (n=244) had a high school degree, and 9.0% (n=53) a secondary school degree, and 0.85% (n=5) had elementary school certification.

The majority of respondents 67.5% (n=396) owned a pet at home, while 32.5 % (n=191) did not. Most of the respondents (88.3%, n=519) were not associated with any environmental association.

The majority of the respondents perceived their interest for Nature as relevant for them (RII= 0.899), and 62.9% (n=370) and 33.8% (n=199) of them rated it as, respectively, very high or high.

The 29.8% (n=175) of the respondents visited zoos and aquaria once each year, 24.4% (n=142) more than once, 3.2% (n=19), and 2.3% (n=19), respectively, once a month or more than once a month.

For the respondents, the most relevant role of zoos and aquaria is environmental education (RII=0.893). Indeed, for the respondents, these zoological institutions play a relevant role also in wildlife conservation (RII= 0.820), wildlife research (RII= 0.854), and in promoting the welfare of wild animals (RII= 0.809) (table 1).

		Frequency and percentage					RII	Item Mean
		Very relevant	Relevant	Slightly relevant	Not at all relevant	I don't know		
How relevant if for you the role of zoos and aquaria regarding	Wildlife conservation	287 48.8%	225 38.3%	43 7.3%	20 3.4%	13 2.2%	0.820	3.28
	Environmental Education	387 65.8%	167 28.4%	19 3.2%	13 2.2%	2 0.3%	0.893	3.57
	Wildlife research	326 55.4 %	205 34.9%	40 6.8%	10 1.7%	7 1.2%	0.854	3.42
	Promoting wildlife welfare	303 51.5%	182 31.0%	59 10.0%	27 4.6%	17 2.9%	0.809	3.24

Table 1 Role of zoos and aquaria in wildlife conservation, environmental education, wildlife research, and in promoting the welfare of wild animals according to respondents.

According to the respondents, the most significant negative consequences caused by the COVID-19 pandemic to zoos and aquaria were economic (RII=0.852).

Respondents perceived as relevant also the impact that the pandemic had on the promotion of scientific knowledge of species hosted in zoos and aquaria (RII= 0.730) and environmental education activities (RII= 0.787). The majority was aware of a lack of public support for zoos and aquaria during the pandemic (42,5% strongly agree, and 41,0% agree) and that the government policy responses did not consider the specificities of zoos and aquaria that host animals.

In table 2 are reported the results of the specific items regarding the consequences of the COVID-19 pandemic on different aspects related to zoos and aquaria.

	Frequency and percentage					RII	Item Mean
	Strongly agree	**Agree**	**Disagree**	**Strongly disagree**	**I don't know**	**RII**	**Item Mean**
Zoos and aquaria, and the people who work there, have been economically very negatively affected by the COVID-19 pandemic	353 60.0%	195 33.2%	3 0.5%	0 0%	37 6.3%	0.852	3.41
The promotion of scientific knowledge of the species hosted in the zoo has been affected by the pandemic	205 34.9 %	270 45,9%	44 7.5 %	0 0%	69 11,7 %	0.730	2.92
The pandemic resulted in a medium/long-term adverse effect on Nature conservation interventions	158 26.9 %	233 39.6%	92 15.6%	9 1.5%	96 16.3%	0.648	2.59
Environmental education activities have been affected by the pandemic and related restrictive measures	233 39.6%	278 47.3%	41 7.0%	4 0.7%	32 5.4%	0.787	3.15
There was a lack of public support for zoos and aquaria during the pandemic	250 42.5%	241 41.0%	36 6.1%	0 0 %	61 10.4%	0.763	3.05
The pandemic has increased fears that animals can transmit diseases (COVID-19 and/or others)	137 23.3%	163 27.7%	185 31.5%	34 5.8%	69 11.7%	0.613	2.45
The pandemic has increased consideration of the social role of zoos and aquaria	85 14.5%	172 29.3%	175 29.8%	18 3.1%	138 23.5%	0.520	2.08
Government policy responses to the pandemic took into account the specific situation of zoos and aquaria	114 19.4 %	60 10.2 %	213 36.2 %	46 7.8 %	155 26.4 %	0.471	1.88

	Frequency and percentage					RII	Item Mean
	Very significant consequences	Significant consequences	Few consequences	No consequences	I don't know	RII	Item Mean
Animal welfare	92 15.65%	234 39.80%	75 12.76%	116 19.73%	71 12.07%	0.568	2.27
Environmental education	141 23.98%	268 45.58%	92 15.65%	32 5.44%	55 9.35%	0.673	2.694
Wildlife conservation	105 17.86%	250 42.52%	97 16.50%	55 9.35%	81 13.78%	0.603	2.413
Scientific research on wildlife conservation	107 18.20%	260 44.22%	102 17.35%	38 6.46%	81 13.78%	0.616	2.466

Table 3. Respondents' opinions on the consequences of the COVID-19 pandemic on different activities of zoos

Finally, when asked for how long the consequences of the pandemic could last, the respondents answered: 18.7 % (n=110) "They will cease within 6 months of the end of the pandemic"; 42.3% (n=249) "They will cease within 1 year of the end of the pandemic"; 30.3% (n=178) "They will cease within 5 years of the end of the pandemic"; 5.4% (n=32) "They will cease within 10 years of the end of the pandemic"; 3.2% (n=19) "They will last more than 10 years".

DISCUSSION

Understanding public perceptions of the impact of the COVID pandemic on zoological institutions is significant to decide what can be done to engage the public in similar cases in the future. According to scientists, pandemics caused by the spillover of viruses from animals will occur more often in the future, mainly due to the disruption of ecosystems and the increased likelihood of interaction between wildlife and humans. In February 2020, WHO Director-General Ghebreyesus (WHO, 2020d) mentioned 216 disease outbreaks that WHO was combatting around the world, of which COVID-19 was just one. Scientists are working to systematically evaluate novel wildlife-origin viruses in terms of their zoonotic spillover and spread potential (Grange et al., 2021). Therefore, zoological institutions should also start to think about how to manage a "continuum of pandemic phases" (WHO, 2017) from the point of view of "safety concepts" (Lindhout & Reniers, 2020). They have to think about how to be able to continue providing entertainment and education to their audiences and raise awareness and funds to support field projects and habitat protection also during these difficult phases.

Zoos play a relevant social role in environmental education and wildlife protection, and also in environmental education and, therefore also, to prevent future spillovers. They can teach people to live safely with wildlife, and this can prevent future spillovers, and the same conserve species often stigmatized as viruses reservoirs, that are essential to our life on the planet (Grange et al., 2021). These institutions play a fundamental role in encouraging visitors to care for wild animals. The empathic relationship they establish with the animals exhibits er care for the zoo animals and, in turn, for their wild counterparts and for the ecosystems in which these animals live (Clayton et al., 2009). However, the result of results of the Survey shows that only a minority think that the COVID-pandemic has increased the social role of zoos and aquaria, while many don't know. However, many respondents think that it had a great impact on wildlife conservation.

Conclusions

Our study showed that the public considers the role of zoos and aquaria very important for environmental education, wildlife research, and wildlife conservation. The respondent perceived as relevant to the consequences of the COVID-19 pandemic on the activities of environmental education and wildlife research. The respondents were aware that zoos had a significant negative economic impact due to closure and periods of restrictions. They also perceived a lack of public support and attention from the Government towards these institutions. This shows that the public was aware that the zoo workers had been left alone at this difficult time. This may suggest that these persons, if better engaged, could have supported the zoos and aquaria more during the pandemic, but this point should be investigated furthermore.

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GENERAL DISCUSSION

During this Ph.D., I applied tenets of conservation ethics to analyze new challenges and innovative approaches in wildlife conservation. Conservation ethics can be configured as applied ethics and is centered on the analysis of what is best to do and how to act to safeguard wildlife and ecosystems. It allows the application of ethical tenets to procedures of wildlife conservation projects taking into account the values and interests of the animals, environment, and men involved. In doing so, ethical conservation allows conservationists to be able to have an immediate and comprehensive assessment of the ethical issues at stake. Although the enterprise of wildlife conservation is inherently ethical, it involves different stakeholders that may have different values and interests. Therefore, the analysis of the acceptability of these wildlife conservation projects cannot be focused only on the preservation and rehabilitation of a particular species, but it necessitates a holistic focus on all the values and interests at stake. Conservation greatly benefits from clearly articulated and widely applied ethical tenets that highlight and analyze the values and interests of all stakeholders. A lack of attention to these ethical aspects can be detrimental to the ethical acceptability of conservation projects, even if they have commendable goals.

In the first part, I applied tenets of conservation ethics to develop a frame for the development of a tool that can be used for the Ethical Review Process (ERP) of wildlife conservation projects applying assisted reproduction technologies, that use natural gametes, and advanced reproductive technologies, that use in-vitro gametes. ERP is a critical reasoning process based on tools and methodologies; it helps to highlight ethically relevant issues, revises existing policies and choices, provides advice, and allows consistency and transparency in communication with institutions and with the public (de Mori et al. 2018).

Conservation projects that use assisted reproduction technologies and, especially, advanced reproduction technologies on wildlife are potent tools in the toolbox of scientists but, at the same time, present various ethical challenges.

During my Ph.D., I worked with the ethicist of the ethical team of BioRescue and with the other scientist of the consortium with a continuous and re-iterative process to shape a framework for the ERP of procedures proposed in the project aimed to save critically endangered northern white rhinoceroses. The development of the ERP for the project started unpacking and analyzing the values and interests of the different stakeholders involved in the first part of the project, the ovum-pick from the two living females of this species: Najin and Fatu. To this aim, it was used an ethical tool: the ethical matrix. The ethical matrix was originally created to assess ethical issues arising from the use of biotechnologies in agriculture and has been adapted to wildlife conservation by Biasetti et al. (2021). The ethical matrix adapted for wildlife conservation analysis includes three categories of potential stakeholders: ecological entities, individual animals, and people. In our case, the ethical matrix presented for ovum pick in northern white rhinoceroses included the following stakeholders: biodiversity, the rhinoceros females subjected to the procedure, and the people involved at any level in the process. The frame of the ethical matrix proposed for the ovum pick-up can also be applied to other ART and aART procedures. Although ERP requires wider ethical analysis, the ethical matrix proposed can help conservationists for a more balanced approach in evaluating complex moral scenarios where different needs, interests, and ethical concerns may conflict.

The values and interests of each stakeholder highlighted through the ethical matrix were integrated into an ethical assessment tool (ETHAS) described in the second part of the first section. The prototype of ETHAS was developed on the ovum-pick procedure. The general frame of ETHAS can be customized for each procedure involved in the application of ARTs to conservation wildlife projects. ETHAS was based on scientific literature, national and international legislation, ethical tenets, and stakeholders' values and interest. The tool consists of two mutually integrated checklists, the Ethical Evaluation Sheet (EES) and the Ethical Risk Assessment (ERA), and combines an ethical risk assessment with an ethical evaluation of the procedures. The tool integrates risk assessment (general, ethical, welfare), pain/distress/welfare evaluation, harm–benefit analysis, and the 3Rs tenet. Considering all these aspects together, the

tool has the potential to make an overall assessment of the procedure and, eventually, help in the detection of problematic issues than using only one of these approaches separately.

During my Ph.D. project, I then proceeded to create ETHAS for the in-vitro laboratory procedure and for the biomolecular procedure used to produce in-vitro gametes from fibroblast or other somatic cells after being reprogrammed in induced pluripotent stems cells (iPSCs). ETHAS applied to the assessment of IVF laboratory procedures with natural gametes resulted "totally acceptable". These procedures are well-assessed in farm animals, such as horses, that are evolutionarily related to rhinoceroses (Price et al., 2009). Therefore the technologies optimized for these animals can be applied, with some adaptation, to rhinoceroses and, indeed, they have already produced 14 viable embryos of northern white rhinoceroses (BioRescue).

The ETHAS assessment of the procedures for iPSC and in-vitro gametes generated a result of "Acceptable with mitigation". These procedures are still at an early stage of optimization. However, the outcomes of the scientific knowledge gained can be of great relevance for mammalian conservation projects. Furthermore, this approach can generate, thanks to meiosis, an enormous variety of new genotypes by reshuffling existing diversity through chromosome reassortment. During our evaluation with ETHAS, we assessed that the use of fibroblasts from cryopreserved tissues of a now-dead northern white rhinoceros, Nabire, for the development of the iPCS procedures was acceptable as the specimens of this individual cannot be used in the future to create newborns as the individual had an altered number of chromosomes (n=81). However, an individual with aneuploidy, i.d. Najin, reproduced naturally and gave birth to an individual, Fatu, with a normal number of chromosomes (n=82). The procedures for producing the iPCS, a first step in the northern white rhinoceroses in-vitro gametogenesis, proved to be safe in the methods and for the purposes for which they were conducted, and scientists of the BioRescue consortium in 2022 succeeded in producing the first iPCS from northern white rhinoceros fibroblasts (Zywitza et al., 2022).

In its application to all the procedures assessed, ETHAS has shown to be able to positively contribute to the process of refinement and optimization. It allowed researchers to reflect on the

procedure, and to possible responsible implementations. Additionally, it helped in promoting open and transparent communication among the conservation project partners.

Assisted reproductive technologies will play a role in future conservation ex-situ projects. Even if the ultimate goal is to produce stable wild populations, for the moment, many endangered species are kept ex-situ in conservancies or other zoological facilities.

During my Ph.D., I worked on developing a zoo ethical evaluation tool (ZERS) tool to assess visitors' opinion on the reputation of zoos, especially regarding the ethical aspects of their reputation. Originally established as places of entertainment and display of rare animals, over the years, zoos have progressively assumed active and prominent positions in wildlife research and biodiversity conservation, supporting an integrated approach to species protection, embracing the One Plan Approach to Conservation (Byers et al. 2013, IUCN). However, to be trusted and effective in their mission, these institutions must act ethically towards all their stakeholders and have a good reputation among them. The level of general awareness of the fairness and ethicality of the zoo's actions can be measured by the level of the public reputation of the zoo. In this work, the main drivers that can impact a zoo's reputation were identified, and through a survey, the opinion of zoo visitors was evaluated on each of them. The results showed that there is a direct correlation between zoo familiarity and visitors' age with emotional appeal and ethical responsibility. These findings suggest that familiarity with zoos, especially when cultivated over the years, creates an emotional bond with these institutions that increases confidence that zoos act with ethical responsibility, thus improving their reputation. We also found that emotional appeal correlates with zoo familiarity; these suggest that positive emotions generated by a visit to a zoo can create a familiarity bond with that institution that will drive the visitor to revisit that institution or even visit other zoological institutions. Finally, ZERS results showed a positive—even if moderate—correlation between ethical responsibility and emotional appeal, revealing how relevant positive emotions experienced during a visit can influence the visitor's opinion about the reputation of that zoo as an ethical institution. Reliability and validity analysis of the first trial of ZERS showed coherent and consistent evidence of its usefulness in

assessing the opinion of zoo visitors on the critical drivers that can determine the reputation of zoos on specific aspects of their activities and their ethical reputation. However, there are some study limitations to take into consideration. First, it was tested only in one zoological institution in Italy and one in Germany, and the results obtained cannot be representative of the opinions of the entire reference population. Second, for validating the questionnaire, it will be crucial to test it also on zoo visitors in zoos of other countries will be for the development of the tool. Finally, as reputation is a multidimensional construct that reflects the unique dimensions on which individual stakeholders base their judgments of an institution (Fombrun et al., 2000), it could be useful in the future to improve the tool in a way that may include the opinions also of other stakeholders (e.g., zoo workers zoological operators, environmentalists, local authorities, children, etc.).

After the COVID-19 outbreak, wildlife conservation had to face new, unexpected challenges. During my Ph.D. I worked to assess the consequences of the new challenges to wild animals, such as bats, caused by the COVID-19 outbreak, applying tenets of ethical communication to analyze how the online news on bats was framed.

Conservationists have to deal with various stakeholders, including local communities. The opinion of local populations can be essential to the success of a conservation project, especially if it involves animals considered dangerous, such as large carnivores, or terrifying and disease-transmitting, such as bats, and these conflicts can be exacerbated by informal communication.

Ethical communication in conservation should be based on three aspects: be reflexive, engage responsibly, and consider the power (Gregg et al., 2022). Nevertheless, people that share news do not follow these tenets, often spreading and overemphasizing the most alarming news stories. The media play an important role in shaping perceptions of wildlife-related risks. For many people, who rarely have contact with wild animals, media represents the only way to form an opinion about them. Moreover, public knowledge and understanding of infectious diseases are based on information released by mass media (Evensen and Clarke, 2012) and how the information is framed influences the perception of the risk level.

The COVID-19 pandemic showed the vulnerability of our society to virus spillover from wild animals. In the first phase of the pandemic, online news often communicated uncertainties about the possible reservoir of the viruses or the possible transmission from animals to humans and vice versa. Rapid information to the public is essential to avoid the further spreading of a dangerous virus like SARS-CoV-2, but such information can be detrimental to the conservation of the blamed species. Since the beginning of the pandemic, bats have been blamed for being the original reservoir, possibly jeopardizing bat conservation efforts.

A recent study by Lu et al., 2023 on the cognitive-emotional pathway concerning stigmatized species, such as bats, tried to understand the role of messages and psychological factors in influencing attitudes toward these animals. The results show that when there are uncertainties associated with the causal link between infectious diseases and wildlife, it is better to communicate such uncertainties. However, the way they are framed can greatly impact readers opinions and cause persecution of already vulnerable species, such as bats.

The results of our research also showed that, after this phase of uncertainty, few months after the pandemic, a significant increase in conservation messages appeared in the news. Maybe thanks to correct communication, very little sensationalistic news on bats were found.

The discussion among the team researchers during our research study allowed the highlighting of key points for effective communication in conservation.

Conservationists play a key role in wildlife science communication, and they can use strategic and persuasive messaging as part of their 'toolbox', but it is important that this is done with openness, transparency, and accountability by providing clear information and contact details (Gregg et al., 2022). They must avoid technical jargon and explain risks with simple examples. They should increase scientific science dissemination in public events, in schools, etc., to demystify how bat diseases are transmitted and give information on how to avoid the transmission, and, at the same time, highlight the importance of these animals' conservation and their ecological role.

However, communication is a multilevel science that involves different stakeholders, such as journalists and audiences. News should be reported in a non-sensationalist way, based on facts and scientific objectivity, never citing myths and legends and with the help of scientific experts to dissipate misinformation. Also, the audience plays a relevant role and should behave as active message receivers checking the accuracy of the information read (fact-checking) with a critical sense, verifying the author and source of information, checking the date, checking the news from multiple media sources, and contacting experts to provide more information in case of relevant doubts.

The COVID-19 pandemic has greatly restricted human activities, and zoos were suddenly empty of their visitors. During this Ph.D., a survey was used to assess the public perception of the difficulties these institutions were facing. The results showed that people were aware of the negative economic impact of the lockdown and other restrictions decided by the Italian government after the COVID-19 outbreak (62.9 % very much agree and 31.1% agree), and this was for the respondents the most relevant consequences of the pandemic on zoos (RII= 0.865). According to respondents that environmental education activities (38,5 % very much agree and 47,9%; agree; RII= 0,778) and promotion of scientific knowledge of wildlife (36,4 % very much agree and 42,7 %; agree; RII= 0,778) were negatively impacted by COVID-19 outbreak. Additionally, many respondents thought there was a lack of public support for zoos and aquariums during the pandemic (40,9 % very much agree and 39,9 %; agree; RII= 0,744).

The analyses of public perception of the impact of these restrictions on zoological institutions can help these institutions to find strategies to understand what can be done to better engage the public in case of new, unexpected adverse events, such COVID-19 pandemic. This can be relevant for the future because, according to researchers, without drastic changes in the way humans manage nature, global epidemics like COVID-19 will become more common (UNEP and ILRI, 2020).

CONCLUSIONS

During my Ph.D. I worked on several independent projects, often working side-to-side with scientists of different disciplines, such as zoology, veterinary, and psychology, to analyze the different ethical issues arising from wild conservation. I used tenets of conservation ethics to develop a tool for the assessment of projects using assisted reproduction technologies in wildlife conservation projects. The frame of the tool proposed is customizable for other ART and aART procedures. The tool is undergoing revision to create a handy, easy-to-use tool that conservationists can use for a self-assessment of the ART and aART procedures from the planning through all their projects.

As zoological institutions play a fundamental role in ex-situ I studied the drivers that can reputation among the public, jeopardizing their educational and conservational efforts.

I worked on the development of a tool for the zoo's ethical reputation, to assess the opinions of zoo visitors on this specific aspect of these drivers. Similar tools are well established for the evaluation of the reputation of other corporations, but, to our knowledge, there are no similar tools to evaluate the reputation and ethical aspects of zoological institutions. However, ZERS can help a zoo or zoological association to evaluate how much the public perceives their commitment to animal welfare, environmental education, and wildlife conservation. Additionally, it can help highlight critical issues and implement strategies to improve them. By addressing them, zoos can not only increase people's trust but, reflecting on measurable parameters, they can be encouraged to operate as ethical institutions, "ethical arks" committed to advancing higher standards and practices towards all their stakeholders.

Finally, I worked on the analysis of new, unexpected challenges to wildlife conservation caused by the COVID-19 outbreak, analyzing how news online on bats was framed and how this could jeopardize bat conservation and the level of awareness of people of the difficulties that zoological institutions had to face.

In all these works, I tested the relevance of conservation ethics for the development of ethical review processes and ethical tools that can help highlight and unpack the ethical issues arising

from wildlife conservation and to evaluate different ethical aspects that can impact wildlife conservation.

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ATTACHMENTS

ATTACHMENT N.1

UNIVERSITÀ DEGLI STUDI DI PADOVA

DEPARTMENT OF COMPARATIVE BIOMEDICINE AND FOOD SCIENCE

Protocol number _____

Biomolecular Laboratory Procedures

ETHICAL EVALUATION SHEET

Bio*Rescue* Ethical Team

Ethics Laboratory for Conservation, Veterinary Medicine and Animal Welfare

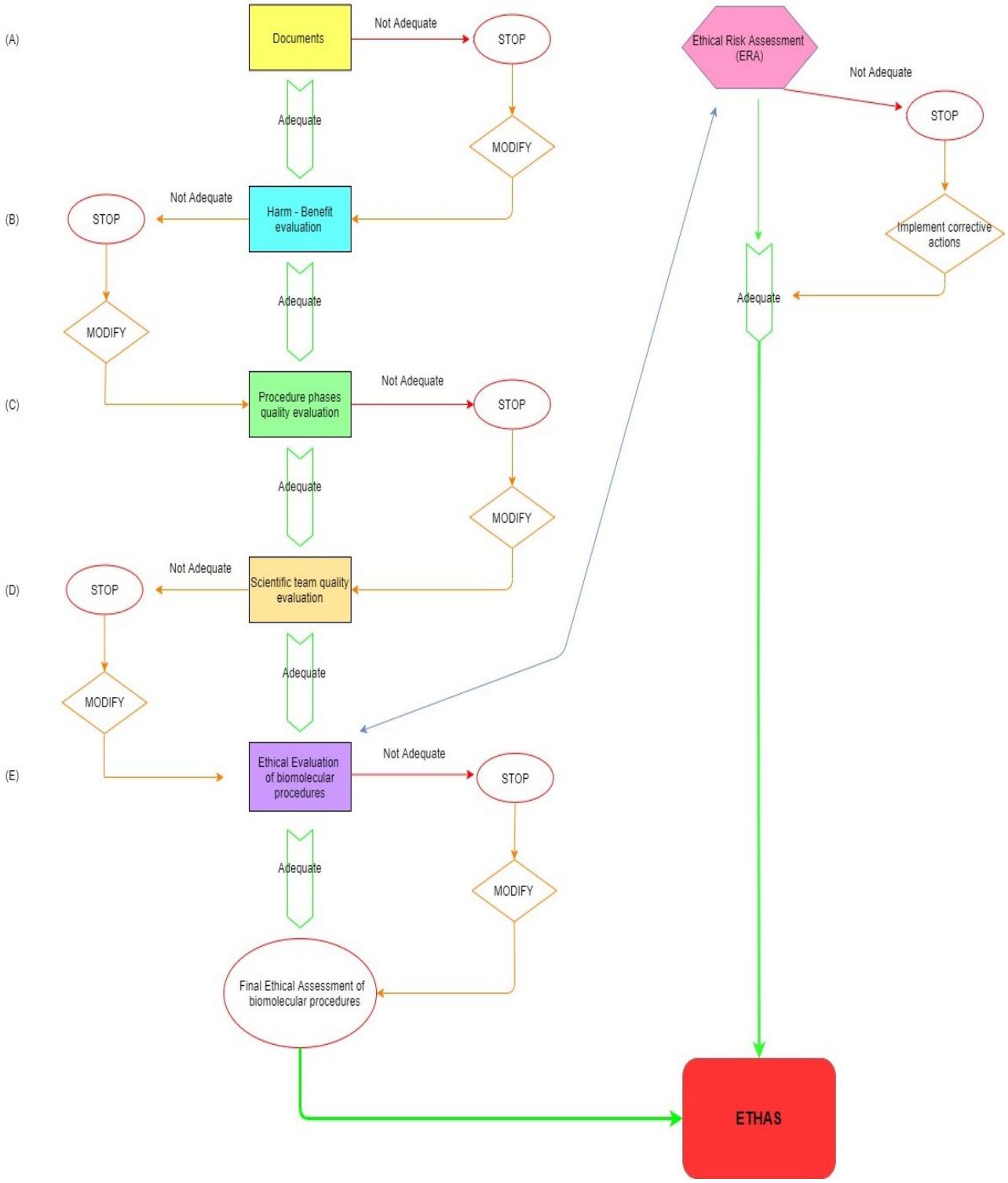

Figure 1: Flow chart for the Biomolecular Laboratory Procedures Ethical Evaluation (from A to E).

A	DOCUMENTS			
				If necessary, please give details
1.	Have all the stem cells and gametes generation protocols been planned in compliance with national and international regulations?	YES	NO	…… …….. …… ……
2.	If the procedures of the biomolecular laboratories are part of a project that involves low and/or lower-middle-income countries, have the potential ethical issues (e.g., *"exploitation of participants, exploitation of local resources, risks to workers & staff)* arising from the research-related activities been adequately evaluated?	YES	NO	…… …….. …… …… …… ……..……. ……
3.	If animal specimens of low and/or lower-middle-income countries are involved, have documents showing compliance with Nagoya Protocol on access and benefit-sharing been submitted to the competent authority? *Any use of local resources (especially animal and/or human tissue samples, genetic material, and live animals) must show respect for cultural traditions.*	YES	NO	…… …….. …… …… …… ……..……. ……
4.	If the procedures of the biomolecular laboratories are part of a project that involves low and/or lower-middle-income countries, are benefit-sharing measures been planned? a) Benefit sharing measures b) Responsiveness to local population needs *Any use of local resources (especially animal and/or human tissue samples, genetic material, and live animals) must show respect for share benefits (i.e., also benefit local participants and their communities, involve local workers – as equal partners – and respond to local worker's needs).*	 YES YES	 NO NO	…… …….. …… …… ……..……. ……
5.	If the procedures of the biomolecular laboratories are part of a project that involves low and/or lower-middle-income countries, are local researchers included, wherever possible, throughout the process (including in study design, study implementation, data ownership, intellectual property, and authorship of publications)?	YES	NO	…… …….. …… …… …… ……..……. …… ……
6.	Have export/import licenses to transfer biomaterial been obtained	YES	NO	…… ……..

7.	Do the laboratories involved have an accreditation? (*e.g.,* ISO accreditation, etc.) If yes, give details. ……	YES	NO	…… ……..….. …… …… ……
8.	Has ethical approval been obtained? If yes, give details. ……	YES	NO	…… ……..….. …… …… …… ……..…… …… ……
B	**HARM - BENEFIT EVALUATION**			
B1	**Benefit evaluation**			
				Detail when nedeed
9.	Are these biomolecular procedures (reprogramming somatic cells into induced pluripotent stem cells, iPSCs, and *in-vitro* gametogenesis, IVG) part of a novel wildlife conservation strategy?	YES	NO	…… ……..….. …… …… ……
10.	Will the application of these biomolecular procedures improve efforts and the advancement of reproductive scientific knowledge?	YES	NO	…… ……..….. …… …… …… ……..…….
11.	Could the application of these biomolecular procedures, as part of a novel conservation strategy, have a positive impact on the natural habitat in the future?	YES	NO	…… ……..….. …… …… …… ……..…….
12.	Will the scientific improvements obtained from these biotechnologies' development positively impact other scientific fields?	YES	NO	…… ……..….. …… ……
13.	Will the development and application of these biomolecular procedures improve the professional training of laboratory personnel?	YES	NO	…… ……..….. ……
14.	Is the scientific knowledge achievable from the development and application of these biomolecular	YES	NO	…… ……..….. ……

	procedures relevant to advanced assisted reproduction for mammals?			…… …
15.	Does the application of these biotechnologies represent a milestone in critically endangered species' reproduction?	YES	NO	…… …… ……..……
16.	Could the application of these biotechnologies have positive side effects on the species involved?	YES	NO	…… …….. …… …… …
B2	**Harm evaluation**			
17.	Do the applications of these biotechnologies or part of them have no adverse side effects on the health of the species involved?	YES	NO	…… …….. …… …….
18.	Do the biotechnologies result in no modification of the genome of the species?	YES	NO	…… …….. ……
19.	Have the possible adverse effects on the specimens that may happen during the following steps been adequately considered? a) iPSCs reprogramming b) *In vitro* gametogenesis c) *In vitro* fertilization with artificial gametes d) Embryos, obtained by artificial gametes development e) Specimens long-term cryopreservation	 YES YES YES YES YES	 NO NO NO NO NO	…… …….. …… …… …… ……..…
20.	Have a) and b) been adequately considered and discussed among the project partners before applying these biotechnologies? a) Public opinions b) Scientists opinions	 YES YES	 NO NO	…… …….. …… …… …… ……..…… …

21.	Have the possible adverse effects on public and scientist opinions of the unfortunate event that something goes wrong on the cells, gametes, or animal health due to the application of biotechnology been adequately considered and discussed among the project partners?	YES	NO	…… ……..….. …… …… …… ……..…
	PROCEDURE PHASES QUALITY EVALUATION			
C1	**Pre-screening considerations**			
22.	Have the quality of these innovative biotechnologies for a) and b) been already tested with success <u>on other mammals</u> (i.e. murine models, *etc.)* in other related studies? a) Somatic cells reprogramming into iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..…… …… …… …… ……..…
23.	Have the quality of these innovative biotechnologies for a) and b) been already tested with success <u>on wild animals</u> in other related studies? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..…… …… …… ……..…… ……
24.	If these innovative biotechnologies have already been used with success in murine models, have the necessary species-specific adaptations been considered for a) and b)? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..…… …… …… ……..…… ……..……
25.	Has the opportunity to modify any step of the protocol for a) and b) ever been considered to improve the procedure for the specimens of this species? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..…… …… ……
26.	Are the project partners committed to eventually publishing also critical events so that they could be avoided in future projects?	YES	NO	…… ……..…… …… ……

27.	Has it been planned to share and publish relevant data on the procedures refinement of these biotechnologies?	YES	NO	…… ……..…… …… ……
C2	**Procedural steps evaluation**			
				Details when needed
28.	Have protocols to produce iPSC of the endangered species been tested and evaluated in other related species?	YES	NO	…… ……..…… …… ……
29.	Have protocols in-vitro oogenesis of the endangered species been tested and evaluated in other related species?	YES	NO	…… ……..…… ……
30.	Have protocols in-vitro spermatogenesis of the endangered species been tested and evaluated in other related species?	YES	NO	…… ……..…… …… …… ….
31.	Has the method used to select egg fertilization been optimized in other related species?	YES	NO	…… ……..…… …… …… …
32.	Has the in-vitro embryo development protocol been tested and evaluated in other related species?	YES	NO	…… ……..…… …… ……
C3	**3Rs evaluation**			
Replacement				
33.	Have alternative procedural steps or parts of them been considered for the execution of a), b), and c)?			…… ……..…… …… …… ….
	a) Somatic cells reprograming into iPSCs	YES	NO	
	b) *In vitro* oogenesis	YES	NO	
	c) *In vitro* spermatogensis	YES	NO	
34.	Is it possible to replace the medium extracted from other animals with synthetic ones? (e.g. *rhinoceros estrum serum for embryos maturation, BSA*, etc.)	YES	NO	…… ……..…… …… ……

35.	Since for in-vitro gametogenesis, it is necessary to have tight interactions between gametes and the gonadal environment, is it possible to replace the cells used to reconstruct *in vitro* a) and b) with cells of farm/laboratory animals? a) Ovary b) Testis	 YES YES	 NO NO	…… …… …… …… …… …..…… …… ……
36.	Is it possible to optimise the procedures on not endangered related species?	YES	NO	…… ……..…… …… ……
37.	Have other preservation methodologies been evaluated for a more efficient long-term biomaterial conservation?	YES	NO	…… ……..…… …… ……
Reduction				
38.	Have a), b), and c) procedures been optimized so that a minimum number of collected specimens will be used? a) Somatic cells reprogramming into iPSCs b) *In vitro* oogenesis c) *In vitro* spermatogenesis	 YES YES YES	 NO NO NO	…… ……..…… …… …… ……..…… ……
39.	Will the gametes or embryos obtained be shared with other scientific groups?	YES	NO	…… ……..…… …… ……
40.	Could the specimens that cannot be used for obtaining newborns (e.g., a surplus of spermatozoa, oocytes, and embryos not adequately developed) be used for other studies?	YES	NO	…… ……..…… …… …… ……..…
Refinement				
41.	Have biomolecular laboratory procedures ever been applied to other animal specimens of other species so that any possible risk has already been evaluated, analyzed, and minimized?	YES	NO	…… ……..…… …… ……
42.	Have alternative procedural steps or part of them that might reduce possible cellular stress of the following specimens been considered? a) Oocytes b) Spermatozoa c) Embryos	 YES YES YES	 NO NO NO	…… ……..…… …… …… ……

43.	Have mitigation actions been included in the protocols to reduce the possible adverse effects that the following specimens might encounter? (i.e., chemical and physical parameters control, *etc*.) a) Oocytes maturation b) Sperm recovery c) Gametes preparation for IVF d) Embryos development e) Specimens long-term cryopreservation	 YES YES YES YES YES	 NO NO NO NO NO	…… ……..…… …… …… …… …… …
44.	To avoid cellular stress, have the external variables (chemicals and physical parameters) been kept within adequate parameters?	YES	NO	…… ……..…… …… ……

D SCIENTIFIC TEAM QUALITY EVALUATION

D1 Team and Teamwork

45.	Are the team's economic resources adequate to deal with following a), b), and c) situations? a) All the procedures of the biotechnologies to produce iPSCs b) All the procedures of the biotechnologies to produce in-vitro gametes c) to overcome problematic situations or emergency management in case of necessity	 YES YES YES	 NO NO NO	…… ……..…… …… …… …… …..……. ……
46.	Has the team already conducted with success a) and b) on mammals (*i.e.,* murine models)? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..…… …… …… ……
47.	Has the team already taken part in relevant and innovative biotechnologies for reproductive procedures?	YES	NO	…… ……..…… ……
48.	Has the team already developed innovative biotechnologies?	YES	NO	…… ……..….
49.	Has the team already produced live newborns from artificial gametes?	YES	NO	…… ……..…… …… ……

50.	In murine models, was the lifespan of the animals obtained by *in vitro* gametogenesis from iPSCs similar to that one of naturally generated ones?	YES	NO	…… ……..… …… ……
51.	Is someone responsible for all steps a) and b) biotechnologies? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..… …… …… ……
52.	Are all the staff members involved in the a) and b) experienced in these biotechnologies? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..… …… …… ……
53.	Has a person been designed to monitor the specimen's conservation status during the long-term cryopreservation procedure?	YES	NO	…… ……..… ……
54.	Is someone responsible for the following steps been assigned? a) Biobanks of the biological material b) Transport of the biological material	 YES YES	 NO NO	…… ……..… …… ……
55.	Is the staff member in charge of transporting the biological specimens trained for this type of material transportation?	YES	NO	…… ……..… ……
D2	**Equipment**			
				Details when needed
56.	Is there adequate equipment necessary for the proper conduction of all a) and b) procedure phases available? a) Reprogramming somatic cells to iPSCs b) *In vitro* gametogenesis	 YES YES	 NO NO	…… ……..… …… ……
57.	Before starting the procedure, has the availability of personal protective equipment (PPE) for all the staff members involved been checked?	YES	NO	…… ……..… ……
58.	Has a control checklist been developed to verify if all the equipment needed for the procedure is available and correctly operating and, if needed, sterilized?	YES	NO	…… ……..… …… ……

D3	Laboratories and BioBanks			
				Details when needed
59.	Are specimens correctly processed in BioBank?	YES	NO	…… …… ……
60.	Has a Risk Assessment of all the laboratory activities been performed?	YES	NO	…… …… ……
61.	If possible, are specimens divided into aliquots and stored in more than one laboratory?	YES	NO	…… ……

E	ETHICAL EVALUATION OF BIOMOLECULAR-LAB PROCEDURE PHASES			
				Details when needed
62.	Has a Biomolecular-lab Ethical Risk Assessment been planned for each laboratory involved in the procedure?	YES	NO	…… …… …… ……
63.	Has a Biomolecular-lab Ethical Risk Assessment been planned each time a procedure is modified?	YES	NO	…… …… ……
64.	In case of a non-satisfied requirement emerging from the Ethical Risk Assessment, will risk mitigation actions be implemented?	YES	NO	…… …… ……
65.	Does the procedure accomplish all the International and National Regulations?	YES	NO	…… …… ……
66.	Has the staff safety been adequately evaluated with dedicated items in the Biomolecular-lab Ethical Risk Assessment?	YES	NO	…… …… ……

67.	The benefits deriving from the procedures' application are:	Low	Medium	High	High
68.	The possibility of the procedures' successful accomplishment is:	Low		Medium	High

69.	The possibility of the procedures' achievement in the scheduled planning is:	Low		Medium	High
70.	The application of the 3Rs principles in the procedures is:	Low		Medium	High
71.	Is it possible to improve the application of the 3Rs?	YES	NO	…… ……..…… …… ……	

FINAL ETHICAL ASSESSMENT OF BIOMOLECULAR-LAB PROCEDURE			
The final assessment of section A, "DOCUMENTS" is: _____/ ____	Acceptable	Partially Acceptable	Not Acceptable
The final assessment of section B, "HARM BENEFIT EVALUATION" is: _____/ ____	Acceptable	Partially Acceptable	Not Acceptable
The final assessment of section C, "PROCEDURE PHASES QUALITY EVALUATION" is: _____/ ____	Acceptable	Partially Acceptable	Not Acceptable
The final assessment of section D, "SCIENTIFIC TEAM QUALITY EVALUATION" is: _____/ ____	Acceptable	Partially Acceptable	Not Acceptable
The final assessment of section E, "ETHICAL EVALUATION OF BIOMOLECULAR PROCEDURE PHASES" is:	Acceptable	Partially Acceptable	Not Acceptable
OVERALL ETHICAL ACCEPTABILITY OF Biomolecular PROCEDURE:	**Acceptable**	**Partially Acceptable**	**Not Acceptable**

Comments,

_____

_____

_____

_____

_____

_____

Ethical B*io*R*escue* Team

Filled by

Place, Date

_____ _____

ATTACHMENT N.2

UNIVERSITÀ DEGLI STUDI DI PADOVA

Protocol number _____

Biomolecular Laboratory Procedures

ETHICAL RISK ASSESSMENT

Bio*Rescue* Ethical Team

Ethics Laboratory for Conservation,
Veterinary Medicine and Animal Welfare

The Ethical Risk Assessment (ERA) allows highlighting the critical points or hazards that could occur during the execution of the biomolecular laboratory procedures for iPSCs generation and in vitro gametogenesis, compromising their accomplishment. The application of ethical principles in the analysis of the risk, together with a risk ethics approach, provides a deeper analysis of the hazards and allows ethical consideration to be part of risk-related decisions. Therefore, ERA provides a base for ethical decision-making and allows the assessment of the ethical acceptability of the procedures. For this purpose, the biomolecular laboratory procedures have been divided into different phases (from phase A to phase I - figure 1). Each phase has been analyzed using a detailed checklist built to identify the safety and ethical hazards. Each item of the ERA checklist is conceptually linked and mutually integrated into an Ethical Evaluation Sheet - EES (the alpha-numerical code of the first column). EES comprises the relevant ethical aspects that need to be detailed in ERA. In case of potential harms or risks identified by the failure to reach a minimum threshold on the ERA score, corrective actions will be planned to mitigate the risks for the success of the procedures. The measures for the risk mitigation consist of implementing activities for reaching an acceptable fulfilment of the requirements defined in ERA or to alleviate the adverse effects that might arise. The "as low as reasonably practicable" principle will be applied. This principle expresses that the risk should be reduced to a level that is low as reasonably practicable unless it can be demonstrated that there is a great disproportion between costs and benefits.

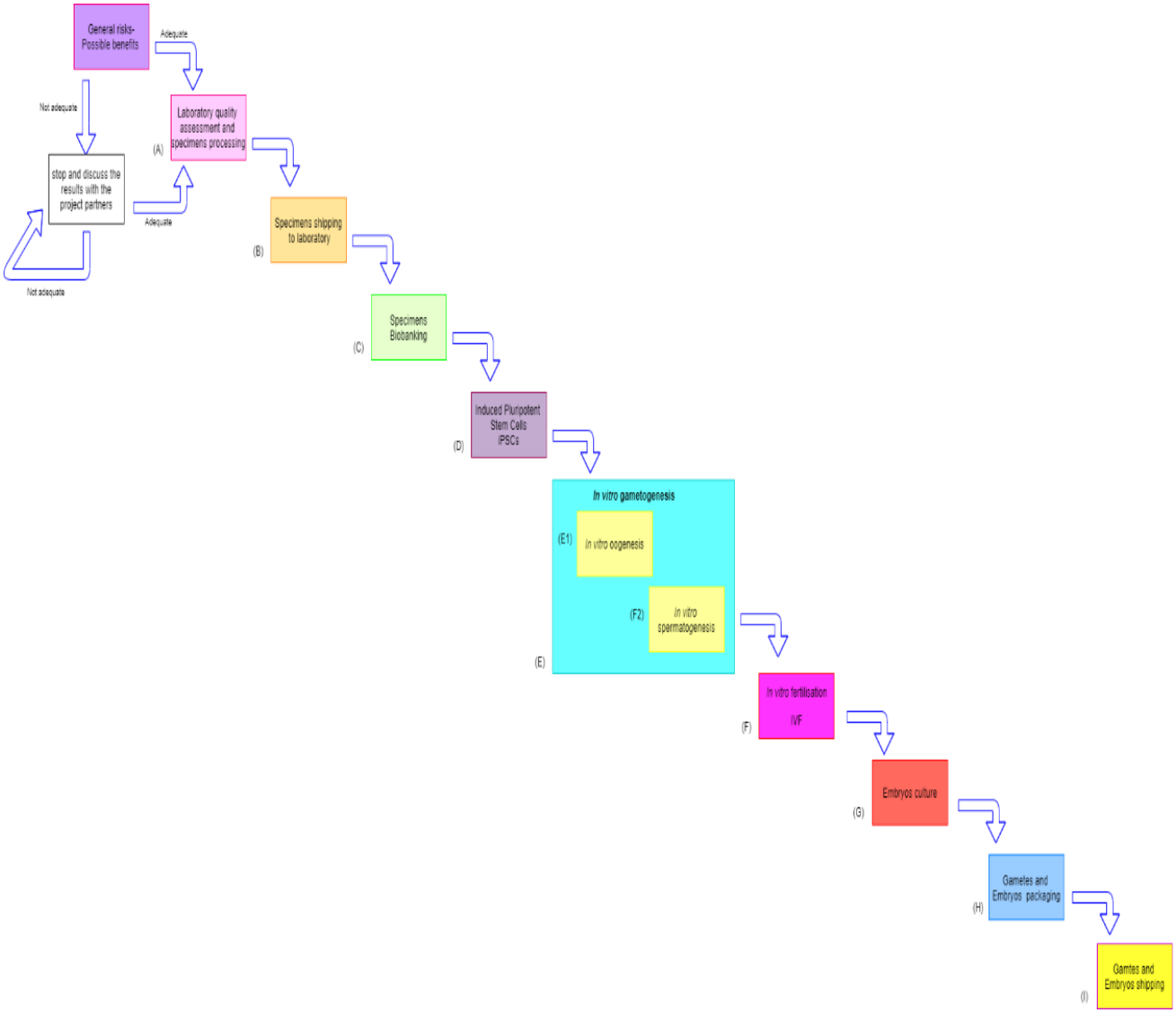

Figure 1: The flow diagram of the Biomolecular Laboratory Procedures ERA phases. The nine phases (phases A-I) in which the biomolecular laboratory procedures have been divided are shown. An initial "General risks- Possible benefits" overall assessment is required before proceeding to the laboratory phases assessme

GENERAL RISKS – POSSIBLE BENEFITS	
Possible Benefits	
• Will the application of this procedure contribute to the improvement of the scientific and technological knowledge on aART (advanced assisted reproduction technologies)?	☐ YES ☐ NO
• Will the application of the procedure contribute to increasing the genetic variability of the species?	☐ YES ☐ NO
• Is the procedure part of a project that can positively impact biodiversity conservation?	☐ YES ☐ NO
Will the application of this procedure permit the use of all biomaterials accessible to scientists (*i.e.,* somatic cells of tissues stored in biobanks) to obtain viable embryos to save endangered species?	☐ YES ☐ NO
Will the application of the procedure have positive effects on the population health of the species?	☐ YES ☐ NO
General Risks	
Have potential harms caused by the application of the procedure that could result in the loss of biomaterial of the endangered species been evaluated?	☐ YES ☐ NO
Have the incidence and severity of possible adverse effects caused by the application of the procedure on the health and well-being of the fetus and/or newborns been evaluated?	☐ YES ☐ NO
Have mitigation strategies been implemented to reduce any adverse effects on the biomaterial of the endangered species during the application of the procedure?	☐ YES ☐ NO
Has it been assessed if tissue donors have transmissible genetic diseases that could seriously affect the health of newborns?	☐ YES ☐ NO
Have the risks of loss or damage of biomaterials of endangered species been discussed among all team members so that everyone agrees that the procedure can be performed?	☐ YES ☐ NO
Number of "NO" in Possible Benefits section: …… Number of "NO" in General Risks section: …… If the number of "NO" in both Possible Benefits and General Risks is equal or minor to 2, you can continue to the following checklist. Otherwise, you have to stop and discuss the results with the project partners.	

PHASE A LABORATORY QUALITY ASSESSMENT, SPECIMENS PROCESSING ASSESSMENT, AND PROCEDURE PLANNING		
EES	***Please answer the items in your knowledge by marking YES or NO. If an item is not relevant to you, leave it blank.***	
A	1. Does the laboratory have an ISO accreditation or any other national authority accreditation? If yes, please specify which one……..	☐ YES ☐ NO
A	2. Has ethical approval/s been obtained for the procedure/s?	☐ YES ☐ NO
A	3. Does the laboratory have an internal Ethical Committee?	☐ YES ☐ NO
A	4. If yes, has internal ethical approval been obtained?	YES NO N/A
D1/A	5. Has the laboratory director developed a policy that confirms a commitment to risk management, assigning authority, responsibility, and accountability at the appropriate levels within the organization?	☐ YES ☐ NO
D3	6. Is risk management considered when planning laboratory procedures, strategies, and activities?	☐ YES ☐ NO
D1	7. Have the necessary resources for risk management been adequately allocated?	☐ YES ☐ NO
D1	8. Does the laboratory have a Risk Team? *(i.e., a group of people responsible for identifying possible risks or adverse events that could occur, evaluating them in terms of occurring probability and severity during regular meetings, and planning guidelines for risk mitigation)*	☐ YES ☐ NO
D1/A	9. Are a), b), and c) present in the laboratory?	
	a) Validated written instructions for each process, including management of adverse events	☐ YES ☐ NO
	b) Warning and accident prevention signs	☐ YES ☐ NO
	c) Continuous training for laboratory biosafety and biosecurity practices	☐ YES ☐ NO
D1	10. Are adverse events or incidents that might occur promptly communicated to the risk team or the responsible for the laboratory?	☐ YES ☐ NO
D1	11. Are all risks and actions that need to be executed for risk management promptly communicated to all staff?	☐ YES ☐ NO
D3	12. In case of emergencies, has the laboratory developed plans that describe the actions to be taken for the following a), b), c), and d)?	
	a) Safety of personnel	☐ YES ☐ NO

	b) Protection of all fresh and cryopreserved material	☐ YES ☐ NO
	c) Limitation of damage to equipment	☐ YES ☐ NO
	d) Limitation of damage to specimens' records and data	☐ YES ☐ NO
D1/ D3	13. Are personnel knowledgeable about the specific hazards posed by a), b), c), d), and e)?	
	a) Carcinogens	☐ YES ☐ NO
	b) Teratogens and mutagens	☐ YES ☐ NO
	c) Toxic gases	☐ YES ☐ NO
	d) Neurotoxins	☐ YES ☐ NO
	e) Reactive and potentially explosive compounds	☐ YES ☐ NO
D3/ A	14. Is the access to the biomaterials storage site (biobank building, storage room, cryostorage units, *etc.*) monitored and documented?	☐ YES ☐ NO
D3/ D2	15. Have maintenance, replacement, and calibration (if needed) schedules been planned to ensure the availability and proper functioning of the instruments?	☐ YES ☐ NO
D2	16. Are the following devices adequately monitored and equipped with alarm systems for detecting any out-of-range temperature and/or liquid nitrogen low level?	
	a) Refrigerators	☐ YES ☐ NO
	b) Cryo-storage units	☐ YES ☐ NO
C2/ A/ D3	17. Are the specimens in arrival processed as described in a), b), c), and d)?	
	a) Unpacked in a room separated from the laboratory	☐ YES ☐ NO
	b) Unpacked in biological safety cabinets	☐ YES ☐ NO
	c) Adequately labeled	☐ YES ☐ NO
	d) Registered in a database with all the information related to the specimens	☐ YES ☐ NO
A/ D3	18. Are sanitation and/or sterilization protocols applied for a), b), and c)?	
	a) Equipment	☐ YES ☐ NO
	b) Laboratories	☐ YES ☐ NO
D3	19. Is there a separate room for washing laboratory glassware and autoclaving?	☐ YES ☐ NO
D3	20. Is there a cleanroom separate from the laboratory? *(A facility designed to maintain extremely low levels of particulate matter, such as dust and particles of airborne organisms, and which has controlled humidity and temperature and sterilized materials before being transferred to it)*	☐ YES ☐ NO ☐ N/A
D3	21. Does the laboratory (and the cleanroom, if present) have a), b), c), and d) for research activities involving stem cells?	
	a) Biosafety cabinets	☐ YES ☐ NO
		☐ YES ☐ NO

	b) Centrifuges	☐ YES ☐ NO
	c) Incubators	☐ YES ☐ NO
	d) Microscope	
D2/ D3	22. Are the equipment and surfaces in the laboratory (and in the cleanroom, if present) cleaned as required in a), b), and c)?	
	a) Alternate different sterile cleaning methods	☐ YES ☐ NO
	b) Every day	☐ YES ☐ NO
	c) Routinely tested for microbiological contamination	☐ YES ☐ NO
D2	23. Are all the necessary PPEs available for the personnel at the laboratory entrance (and at the cleanroom entrance, if present)?	☐ YES ☐ NO
A	24. If in vivo procedures are planned, has ethical approval been obtained for using the research animal/s? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
A	25. If in vivo procedures are planned, has it been evaluated if they comply with all national and international regulations? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
C3	26. If in vivo procedures are planned, has the most recent scientific literature been reviewed to examine alternative methods that could provide the same information and/or give the same results? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
C3	27. If in vivo procedures are planned, is a strategy in place to use as few animals as possible? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
C3	28. If in vivo procedures are planned, is a strategy in place to reduce the manipulations of the animals? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
C3	29. If in vivo procedures are planned, is a strategy in place to reduce the animals' stress during manipulations? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
C3	30. If in vivo procedures are planned, have the procedures been optimized to reduce pain and distress or lasting harm that research animals may experience? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
C3	31. If in vivo procedures are planned, and the animal has to be euthanized, is the method chosen the best one to avoid severe suffering? *(Please mark N/A if no in vivo procedure is planned)*	☐ YES ☐ NO ☐ N/A
D1	32. Do the team members commit to a), b), and c)?	
	a) Respect the intellectual property rights of any scientific discovery/procedural improvement that each member might achieve during the application of the procedure	☐ YES ☐ NO

	b) Acknowledge/reference all sources and contributions in future publications	☐ YES ☐ NO
	c) Respect the cultural traditions of other countries involved in the use of resources (especially animal tissue samples, genetic material, live animals, and endangered fauna samples) when applying the procedures	☐ YES ☐ NO
D1	33. Has the public acceptability of in vitro gametes production from somatic cells, through iPSCs creation, been assessed before its implementation?	☐ YES ☐ NO
D1	34. Have the purposes and the pros and cons of obtaining gametes in vitro from somatic cells by creating iPSCs been communicated and discussed with all the staff involved before their implementation?	☐ YES ☐ NO
D1	35. Has adequate communication planning among team members been developed so that everyone supports the strategy chosen to implement the procedures, is aware of and shares the decisions made about potential risks and the strategies planned to mitigate them?	☐ YES ☐ NO
D1	36. Has a communication plan been designed to inform a), b), and c) the results of the procedures?	
	a) Press	☐ YES ☐ NO
	b) Local participants	☐ YES ☐ NO
	c) General public	☐ YES ☐ NO
D1	37. Has the staff been rigorously trained for a), b), and c)?	
	a) Research design	☐ YES ☐ NO
	b) Methodology	☐ YES ☐ NO
	c) Analysis of data	☐ YES ☐ NO
D1	38. Are the differences in age, gender, culture, religion, ethnic origin, and social class among the project partners respected when recruiting staff and during their work?	☐ YES ☐ NO
D1	39. Have fair and equitable actions been included in the contracts or agreements between the staff involved to manage the use, ownership and/or protection of research results under intellectual property rights?	☐ YES ☐ NO
D1	40. Unless otherwise agreed, will the collected data be available to all the staff in a timely, open, transparent, and accurate manner?	☐ YES ☐ NO
D1	41. Will the collected data be communicated to the general public and social media?	☐ YES ☐ NO
PHASE B	**SPECIMENS SHIPPING TO THE LABORATORY**	

A	42. Has a checklist of the documents (certifications, permits, authorization letters) required by the national and international authorities for specimens (cell lines, embryos, tissues, gametes) shipping been drawn up for a) and b)? a) Export b) Import	 ☐ YES ☐ NO ☐ YES ☐ NO
	43. Are samples shipped by courier services certified for the transport of biological samples?	YES ☐ NO ☐ N/A ☐
D1	44. In the case of hand delivery, have the training and certification of the person in charge of the specimens' shipping been assessed?	YES ☐ NO ☐ N/A ☐
C2/ A	45. Is there a protocol for dealing with possible emergencies (e.g., changes in temperature, spills, etc.) during transport?	☐ YES ☐ NO
D2	46. Are the specimens transported into a portable device that can control the temperature chain?	☐ YES ☐ NO
C2	47. Have methods been developed to assess the condition of specimens' packaging before dispatch and after receipt?	☐ YES ☐ NO
C2	48. Is it possible to track the shipment of the specimens at any stage?	☐ YES ☐ NO
C2/ B2	49. Are the specimens preserved from X-ray check control in the airports?	☐ YES ☐ NO
PHASE C	**SPECIMENS BIOBANKING**	
A	50. Is there a database including the following information? a) Date and time of the specimen collection b) Cells donor information c) Relevant history of the specimens (e.g., collection, shipment, etc.) d) Cryogenic vial label information (*e.g.,* number, etc.) e) Cryopreservation method f) Date and time of cryopreservation g) Operator h) Concentration per cryogenic vial (if possible) i) Location (tank, canister) of stored samples in the cryogenic device	 ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO
C2/ A	51. Is it possible to trace all the access (e.g., operator, date, etc.) to the specimens?	☐ YES ☐ NO
C2/ A	52. Are all the specimen handling times documented?	☐ YES ☐ NO
C2	53. Are the specimens adequately labeled with clear, permanent, and durable labels to avoid misidentification?	☐ YES ☐ NO

C2	54. Are the specimens treated to prevent microbial contamination before storage?	☐ YES ☐ NO
C2	55. Are samples analyzed with quality tests before storage? (if possible)	☐ YES ☐ NO ☐ N/A
A	56. Are all biobanks involved in the storage of the specimens certified?	☐ YES ☐ NO
D3	57. If possible, are the specimens of the same cell line separated and stored in a), b), and c)?	
	a) Different storage containers	☐ YES ☐ NO
	b) Different structures/rooms	☐ YES ☐ NO
	c) Different biobanks	☐ YES ☐ NO
A/ D3	58. Is there a periodic inventory of the contents of the biobank, including cross-referencing of specimens with storage records?	☐ YES ☐ NO
A/ D3	59. Is the freezing protocol used for the specimens the best available to preserve them?	☐ YES ☐ NO
PHASE D	**INDUCED PLURIPOTENT STEM CELLS (iPSCs)**	
	If your laboratory does not carry out this phase, please mark the adjacent N/A box and leave the items in this section blank. **N/A** ☐	
C2/ C3	60. Is the method applied to generate iPSCs from somatic cells based on the most updated scientific knowledge?	☐ YES ☐ NO
C1	61. Based on current scientific knowledge, are the protocols applied to produce iPSC the best for the species of interest (or for related species if this is the first application to cells of the species of interest)?	☐ YES ☐ NO
C2/ C3	62. Has the method used to generate iPSCs from somatic cells been optimized for a) and b)?	
	a) Species related to the one subjected to the procedure	☐ YES ☐ NO
	b) Species subjected to the procedure	☐ YES ☐ NO
C2/ C3	63. Are the genes involved in the reprogramming of somatic cells into iPSCs been characterized for a) and b)?	
	a) Species related to the one subjected to the procedure	☐ YES ☐ NO
	b) Species subjected to the procedure	☐ YES ☐ NO
C2/ C3	64. Are the cell culture conditions optimized for the isolation and culture of iPSCs of a) and b)?	
	a) Species related to the one subjected to the procedure	☐ YES ☐ NO
	b) Species subjected to the procedure	☐ YES ☐ NO

C2/ C3	65. To assess the pluripotent state of iPSCs, have a) and b) been evaluated? a) Morphological analysis of cells (*e.g.,* presence of high nuclear-cytoplasmic ratio, chromatin states, *etc.*) b) Gene expression of pluripotent markers	☐ YES ☐ NO ☐ YES ☐ NO
C3	66. To assess the pluripotent state of iPSCs, are techniques used that do not require *in vivo* testing? (*i.e., without the use of laboratory animals for teratoma formation assay, etc.)*	☐ YES ☐ NO
C3	67. Has a standardized *in vitro* assay been developed to evaluate with stringency the pluripotent state of the iPSCs obtained? (*e.g.,* global transcriptomics analysis, formation of embryoid bodies EBs, etc.)	☐ YES ☐ NO
C2/ C3	68. Does the method applied to produce iPSCs use a methodology that avoids or eliminates the integration of exogenous DNA into the cell genome?	☐ YES ☐ NO
C2	69. Are a) and b) of the cell reprogramming procedure used to generate iPSCs of the species of interest consistent with the most recent scientific data of other mammalian cells? a) Efficiency b) Reproducibility	☐ YES ☐ NO ☐ YES ☐ NO
C3	70. If cells do not reach the iPSCs stage, can they be used for another experiment and/or procedural optimization?	☐ YES ☐ NO
B2/ C2	71. Are the iPSCs controlled for the absence of a), b), and c) before storage? a) Endotoxins b) Bacteria c) Viruses	☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO
B2/ C2	72. Are iPSCs analyzed by transcriptome analysis to assess their expression profile and compare it to that of natural stem cells of the species to detect any alterations in gene expression?	☐ YES ☐ NO
B2/ C2	73. Are the iPSCs evaluated with a standardized protocol for a), b), c), and d) before being used to produce in vitro gametes? a) Cellular abnormalities b) Chromosomal abnormalities c) Gene mutations d) Presence of transgenic DNA sequence of the DNA construct used for reprogramming	☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO

PHASE E	**IN VITRO GAMETOGENESIS (IVG)**	
PHASE E1	**IN VITRO OOGENESIS** *If your laboratory does not carry out this phase, please mark the adjacent N/A box and leave the items of his section blank.* ☐ **N/A**	
C1	74. Based on current scientific knowledge, are the protocols applied for the oocyte production from iPSC the best available for the species of interest?	☐ YES ☐ NO
C2/ C3	75. Is all the pathway of the whole process of <u>*in vivo*</u> oogenesis of a), b), and c) well-known?	
	a) Species subjected to the procedure	☐ YES ☐ NO
	b) Species related to the one subjected to the procedure	☐ YES ☐ NO
	c) Other mammals	☐ YES ☐ NO
C2/ C3	76. Are the a), b), and c) phases of the pathway of the <u>*in vitro*</u> oogenesis known in mammals? (*i.e., in murine model*)	
	a) *In vitro* differentiation (IVDi)	☐ YES ☐ NO
	b) *In vitro* growth (IVG)	☐ YES ☐ NO
	c) *In vitro* maturation (IVM)	☐ YES ☐ NO
C2/ C3	77. Are the a), b), and c) phases of the pathway of the <u>*in vitro*</u> <u>oogenesis known in species subjected to the procedure</u>?	
	a) *In vitro* differentiation (IVDi)	☐ YES ☐ NO
	b) *In vitro* growth (IVG)	☐ YES ☐ NO
	c) *In vitro* maturation (IVM)	☐ YES ☐ NO
C2/ C3	78. Has the whole pathway of the <u>*in vitro*</u> oogenesis process already been reproduced with success in other mammals? (i.e., in the murine model)	☐ YES ☐ NO
B2/ C2	79. For *in vitro* oogenesis, will cells without integrated exogenous DNA sequence (i.e., reprogramming vectors, reporter constructs, etc.) be used?	☐ YES ☐ NO
C2	80. Is the frequency of synapsis between homologous chromosomes the same in oogenesis *in vitro* and *in vivo*?	☐ YES ☐ NO
C2	81. Has the chosen method been adequately evaluated (possibly also in comparison to other methods) for its efficiency in creating synapses between homologous chromosomes during *in vitro* oogenesis?	☐ YES ☐ NO
C2	82. Has the chosen method been adequately evaluated (possibly also in comparison with other methods) for its ability to avoid mispairing of homologous chromosomes during *in vitro* oogenesis?	☐ YES ☐ NO
C2		

	83. To assess the quality of the oogenesis *in vitro*, will the a), b), and c) be adequately evaluated?	
	84. Oocytes cytology	☐ YES ☐ NO
	85. RNA transcriptome analysis	☐ YES ☐ NO
	86. Chromosome abnormalities (both numerical abnormalities, such as aneuploidy, and structural abnormalities)	☐ YES ☐ NO
C2	87. Is the efficiency of the oogenesis *in vitro* culture system comparable with that one *in vivo*? (*e.g.,* evaluated in murine model)	☐ YES ☐ NO
C2/ B2	88. Are the expression dynamics of the genes involved *in vitro* oogenesis consistently similar to those *in vivo*? (*e.g.,* evaluated in murine model)	☐ YES ☐ NO
C2/ B2	89. Are transposon transcripts linked to oocyte transcriptional regulation the same during oogenesis *in vitro* as it occurs *in vivo*? (*e.g.,* evaluated in murine model)	☐ YES ☐ NO
C3	90. If it is necessary, is it possible to obtain artificial oocytes also from embryonic stem cells (ECSs)?	☐ YES ☐ NO
C3	91. Since for a complete in vitro oogenesis a tight interaction between oocytes and gonadal somatic cells is necessary (especially in the latter phases of meiosis, follicle formation and oocyte growth), is it possible to use reconstituted ovary (rOvary) to recreate the ovarian environment avoiding the use of living animals?	☐ YES ☐ NO
C3	92. Is it possible to use ovarian tissue of laboratory or farm animals to reconstitute the natural environment for the development of the endangered species oocytes?	☐ YES ☐ NO
C3	93. Is it possible to use for other experiments and/or procedural optimization the cells that, for any reason, were not finally transformed into oocytes?	☐ YES ☐ NO
PHASE E2 IN VITRO SPERMATOGENESIS *If your laboratory does not carry out this phase, please mark the adjacent N/A box and leave the items in this section blank.* ☐ **N/A**		
C1	94. Based on current scientific knowledge, are the protocols applied for the oocyte production from iPSC the best available for the species of interest?	☐ YES ☐ NO
C2/ C3	95. Is the pathway of the whole <u>*in vivo*</u> spermatogenesis process well-known for a) and b)?	
	a) Mammals	☐ YES ☐ NO
	b) Related species	☐ YES ☐ NO
	c) Species subjected to the procedure	☐ YES ☐ NO
C2/ C3	96. Is the pathway of the whole process of spermatogenesis <u>*in vitro*</u> well-known for a) and b)?	
	a) Mammals	☐ YES ☐ NO
		☐ YES ☐ NO

	b) Related species c) Species subjected to the procedure	☐ YES ☐ NO
C2/ B2	97. For *in vitro* spermatogenesis, will cells without integrated exogenous DNA sequence (i.e., reprogramming vectors, reporter constructs, etc.) be used?	☐ YES ☐ NO
C2	98. Is the frequency of synapsis between homologous chromosomes the same in spermatogenesis *in vitro* and *in vivo*?	☐ YES ☐ NO
C2	99. Has the chosen method been adequately evaluated (possibly also in comparison with other methods) for its efficiency in creating synapses between homologous chromosomes during *in vitro* spermatogenesis?	☐ YES ☐ NO
C2/ B2	100. Has the chosen method been adequately evaluated (possibly also in comparison with other methods) for its ability to avoid the mispairing of homologous chromosomes during *in vitro* spermatogenesis?	☐ YES ☐ NO
C2	101. To assess the quality of in vitro spermatogenesis, will the a), b), and c) be adequately evaluated?	
	a) Spermatozoa cytology	☐ YES ☐ NO
	b) RNA transcriptome analysis	☐ YES ☐ NO
	c) Chromosome abnormalities (both numerical abnormalities, such as aneuploidy, and structural abnormalities)	☐ YES ☐ NO
C2	100. Are the expression dynamics of genes involved in vitro spermatogenesis similar to those in vivo? (e.g., evaluated in murine model)	☐ YES ☐ NO
C2	101. Is the efficiency of spermatogenesis *in vitro* culture system comparable to spermatogenesis *in vivo*? (*e.g.,* evaluated in murine model)	☐ YES ☐ NO
C2	102. Is the genes expression during *in vitro* spermatogenesis comparable to *in vivo* spermatogenesis? (*e.g.,* evaluated in murine model)	☐ YES ☐ NO
C3	103. If it is necessary, is it possible to obtain artificial spermatozoa also from embryonic stem cells (ECSs)?	☐ YES ☐ NO
C3	104. Since for a complete in vitro spermatogenesis a tight interaction between spermatozoa and gonadal cells (Sertoli cells) is necessary, is it possible to use an *in vitro* culture of testicular tissue to recreate the testis environment avoiding the use of living animals?	☐ YES ☐ NO
C3	105. Is it possible to use testicular tissues of laboratory or farm animals to reconstitute the natural environment for the development of the spermatozoa of the species subjected to the procedure?	☐ YES ☐ NO
C3	106. Is it possible to use for other experiments and/or procedural optimization the cells that, for any reason, were not finally transformed into spermatozoa?	☐ YES ☐ NO

PHASE F	**IN VITRO FERTILISATION (IVF)** *If your laboratory does not carry out this phase, Please mark the adjacent N/A box and leave the items in this section blank.* **N/A** ☐	
C2	107. Is the fertilization efficiency of the spermatozoa generated *in vitro* similar to that of the spermatozoa generated *in vivo* in a) and b)? a) Other mammals (e.g., murine model) b) In the species subjected to the procedure *(if this is the first application of in vitro generated spermatozoa to IVF in the species of interest, please mark the box N/A)*	☐ YES ☐ NO ☐ YES ☐ NO ☐ N/A
C2	108. Is the fertilization rate of the oocytes generated *in vitro* similar to that of the oocytes generated *in vivo* in a) and b)? a) Other mammals (murine model) b) In the species subjected to the procedure (if this is the first application of in vitro generated oocytes of the species of interest to IVF, Please mark the box N/A)	☐ YES ☐ NO ☐ YES ☐ NO ☐ N/A
C2	109. Before ICSI, are the artificial gametes adequately controlled for the absence of a), b), and c)? a) Endotoxins b) Bacterias c) Viruses	☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO
D2/ C2	110. Is the piezo-driven micromanipulator used the best available that can avoid oocyte damage and improve the fertilization rate? *(e.g., a piezo-stepper that can control the pressure of the intracytoplasmatic injection)*	☐ YES ☐ NO
C2	111. Is ICSI the best fertilization practice with the highest percentage of egg fertilization success?	☐ YES ☐ NO
C2/ C3	112. To improve the fertilization rate, is the maturation time of the oocytes used optimized?	☐ YES ☐ NO
C2/ C3	113. To prevent oocyte damage, are all the steps of oocytes manipulation (cumulus cell removal, sperm injection position in the membrane, etc.) optimized?	☐ YES ☐ NO
C2/ C3	114. To prevent oocyte damage, are hyaluronidase concentration and exposure timing optimized to be kept to a minimum to remove cumulus cells from oocytes?	☐ YES ☐ NO
C2/ C3	115. Is there a technique to improve the low rate of fertilization and cleavage after sperm injection? *(e.g., through electrical activation after sperm injection)*	☐ YES ☐ NO
PHASE G	**EMBRYOS CULTURE** *If your laboratory does not carry out this phase, Please mark the adjacent N/A box and leave the items in this section blank.* **N/A**	

C1	116. Based on current scientific knowledge, is the embryo culture protocol the best available for species subjected to the procedure?	☐ YES ☐ NO
C2/ C3	117. Has the embryo culture protocol been optimized for the species subjected to the procedure?	☐ YES ☐ NO
C2/ C3	118. Has the embryo culture protocol been optimized for species related to the one subjected to the procedure?	☐ YES ☐ NO
C2/ C3	119. Is the timing of medium changing optimized for the species subjected to the procedure?	☐ YES ☐ NO
A/ D3	120. Are all the data related to the status of the embryo adequately recorded?	☐ YES ☐ NO
C1/ D3	121. Do all the manipulations of the embryos take place in an adequate place (in terms of sanification, cleanliness, etc.) and with sanitized/sterilized materials?	☐ YES ☐ NO
C1	122. In previous studies in the murine model (if this is the first application of the procedure to the species of interest), was the development of embryos obtained from in vitro generated gametes comparable to that obtained from natural gametes?	☐ YES ☐ NO
C1	123. In previous studies in the mouse model (if this is the first application of the procedure to the species of interest), was the development of the embryonic adnexa of the embryo obtained from in vitro generated gametes comparable to that of embryos obtained from natural gametes?	☐ YES ☐ NO
C1/ B2	124. In previous studies in the murine model (if this is the first application of the procedure to the species of interest), did a) and b), used in the IFV procedure, produce healthy newborns? a) *In vitro*-generated oocytes b) *In vitro*-generated spermatozoa	☐ YES ☐ NO ☐ YES ☐ NO
C1/ B2	125. In previous studies in the murine model (if this is the first application of the procedure to the species of interest), was the lifespan of the animals produced with a) and b) similar to that of naturally generated ones? a) *In vitro*-generated oocytes b) *In vitro*-generated spermatozoa	☐ YES ☐ NO ☐ YES ☐ NO
C3	126. Is it possible to use for other experiments and/or procedural optimization embryos that, for any reason, are not used to produce newborns?	☐ YES ☐ NO
C3	127. If the creation of chimeric embryos is planned, has the most recent scientific literature been reviewed to examine alternative methods that could provide the same information and/or give the same results? *(Please mark N/A if no creation of chimeric embryos is planned)*	☐ YES ☐ NO ☐ N/A
A	128. If chimeric embryos are created, has it been evaluated whether their creation and use comply with all national and international regulations? *(Please mark N/A if no creation of chimeric embryos is planned)*	☐ YES ☐ NO ☐ N/A

C3	129. If chimeric embryos are created, will the embryo/s be destroyed at an early development stage (compatible with the experiment protocol)? *(Please mark N/A if no creation of chimeric embryos is planned)*	☐ YES ☐ NO ☐ N/A
PHASE H	**IPSCs, GAMETES, AND EMBRYOS PACKAGING**	
A/ D1	130. To carry out the process in line with the relevant national and international regulations, has the staff involved in the specimen packaging been adequately assessed regarding the following aspects? a) Training b) Certification c) Competence	 ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO
C2/ D2	131. Has the packaging of specimens adequately been considered in terms of a) and b)? a) Temperature control b) Incubator volume limit	 ☐ YES ☐ NO ☐ YES ☐ NO
C2/ C2	132. Has a method been developed to adequately mark and label samples to be packaged to identify them clearly?	☐ YES ☐ NO
C2/ A	133. Have different tools (*e.g.*, checklists, etc.) and/or procedures been developed to check the correct packaging?	☐ YES ☐ NO
PHASE I	**IPSCs, GAMETES, AND EMBRYOS SHIPPING from the laboratory**	
A	134. Has a checklist to verify documents (certifications, permits, authorization letters) required by the national and international authorities for the specimens shipping been drawn up for the following details? a) Export b) Import	 ☐ YES ☐ NO ☐ YES ☐ NO
A/ D1	135. Have the training and certification of the person in charge of specimen shipping been assessed?	☐ YES ☐ NO
A/ C2	136. Is there a protocol to be applied to respond adequately to emergencies? (*e.g.,* temperature variations, such as spills, damages, or theft of materials during transportation and any other realistic and foreseeable emergencies)	☐ YES ☐ NO
D2	137. Are the specimens transported into a portable device that can control the temperature chain?	☐ YES ☐ NO
C2/ A	138. Have methods been developed to assess the condition of specimen packaging before dispatch and after receipt?	☐ YES ☐ NO
A/ C2	139. Is it possible to track the shipment of the specimens in any phase?	☐ YES ☐ NO

C2/ B2	140. Are the specimens preserved from X-ray check control in the airports?	☐ YES ☐ NO

Comments,

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I hereby give my consent for the processing of data provided on the Ethical Risk Assessment (ERA) form to be stored, processed, analyzed, and published by B*io*R*escue* project partner for scientific research purposes.

Place, Date

Signature